Biochemical Pharmacology, Vol. 52, 1719–1727, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00523-0

The Effect of Prolactin on Casein Kinase II, MAP Kinase and PKC in Rabbit Mammary Cells and Nb_2 Rat Lymphoid Cells

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ABSTRACT. Prolactin induces milk protein gene expression in rabbit primary mammary cells without any concomitant cell multiplication. Prolactin or other lactogenic hormones is the major inducer of cell division in the rat lymphoid Nb_2 cells. In Nb_2 cells, prolactin also rapidly induces the expression of the c-myc gene, and β-actin and stathmin gene expression is induced more slowly. The possible involvement of casein kinase II (CKII), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) in these process is not well known. The present work was undertaken to evaluate the effect of prolactin on these protein kinases and to determine the possible involvement of these enzymes in the activity of several genes under the control of the hormone. In rabbit mammary cells, prolactin did not alter CKII activity but did transiently stimulate MAP kinase activity. Prolactin also stimulated Ca^{2+}-independent PKC. This effect was visible after 10 min and was maintained for at least 24 hr. Staurosporine, an inhibitor of PKC and of several tyrosine kinases altered Ca^{2+}-independent PKC only moderately. In contrast, GF 109203X, a potent and specific inhibitor of PKC, abrogated almost all PKC activity. Staurosporine, but not GF 109203X, prevented the induction of the casein gene by prolactin. In Nb_2 cells, prolactin induced a slow stimulation of CKII activity. The hormone did not induce MAP kinase activity. Prolactin stimulated Ca^{2+}-independent PKC over periods of 24 hr. GF 109203X, but not staurosporine, inhibited PKC activity, whereas staurosporine but not GF 109203X, inhibited the induction of Nb_2 cell multiplication and the accumulation of c-myc, β-actin and stathmin mRNAs. From these data, it can be concluded that (1) the stimulation of CKII by prolactin in Nb_2 cells is concomitant with cell multiplication: (2) MAPK stimulation is not necessary for prolactin to induce Nb_2 cell multiplication; and (3) PKC is stimulated in mammary and Nb_2 cells, but this stimulation is not required for prolactin to stimulate casein, c-myc, β-actin and stathmin gene expression and Nb_2 cell division. © *1996 by Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1719–1727, 1996.

KEY WORDS. prolactin; casein kinase II; MAP kinase; PKC; casein

Prolactin has many functions, but its mechanism of action has been studied in a limited number of biological systems [1]. For studies at the gene level, essentially three target cells are used: lymphoid Nb_2 cells [2], the pigeon crop sac [3] and the mammary gland [4]. Nb_2 cells are widely used for this purpose because their multiplication is strictly dependent on the presence of prolactin in the culture medium. Similarly, milk protein gene expression in the mammary gland is under the strict control of prolactin.

Previous studies have shown that PKC† may play an important role in the proliferative action of prolactin on Nb_2 cells [5–7]. CKII [8] and MAP kinase [9] are stimulated by different growth factors in different cell types. The possible involvement of these kinases in prolactin action on Nb_2 cells is not known. Tyrosine kinases JAK_2 and Fyn are involved in prolactin action in several of their target cells, including Nb_2 cells [10–16].

Mammary cell growth and differentiation are controlled by several hormones. Prolactin has both mammogenic and lactogenic activity *in vivo* and only lactogenic activity *in vitro* [1]. Mammary explants from mid-pregnant animals have been used extensively to study the mechanism of lactogenic hormones *in vitro*. Isolated epithelial mammary cells can also be used. When cells are completely isolated and cultured on plastic support, they show a moderate capacity to respond to lactogenic hormones. When freshly prepared, cells kept as aggregates or cultured on floating collagen I gel respond intensively to lactogenic hormones. Rabbit mam-

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† *Abbreviations:* CKII, casein kinase II; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-0-tetradecanoylphorbol-13-acetate; MBP, myelin basic protein; 6-DMAP, 6-dimethylaminopurine; 2-AP, 2-aminopurine; PRL, prolactin; MGF, mammary gland factor; St, staurosporine; GF, GF 109203X.

Received 27 March 1996; accepted 3 July 1996.

mary cells are quite sensitive to prolactin alone; insulin and glucocorticoids exert only a moderate amplificatory action. Thus, rabbit primary mammary cells are an excellent biological material to study the mechanism of prolactin action [17]. In a previous study it was shown that several protein kinase inhibitors prevent induction of casein gene expression by prolactin, whereas other inhibitors are without any effect [4, 18]. Recent studies have shown that the protein tyrosine kinase JAK_2 [12, 19] and tyrosine phosphatase [20] play an essential role in the transduction of the prolactin signal to milk protein genes.

The present work was done to evaluate the effect of prolactin on three protein kinases, CKII, MAPK and PKC; the possible involvement of these kinases in the action of the hormone on cell division, on the expression of specific genes in Nb_2 cells and on milk protein gene expression in the mammary cell was also been studied.

MATERIALS AND METHODS

Mammary Cell Cultures

Mammary cell aggregates were isolated from the mammary gland of mid-pregnant rabbits by using collagenase and hyaluronidase. These cell aggregates were seeded on plastic or collagen I gel. After several days in a serum-free and lactogenic hormone-free medium (Ultroser SF, IBF France), cells were deinduced and devoid of casein mRNA. When cells were cultured on collagen I gel, the gel was released from the dish 1 day before the addition of prolactin. All these techniques have been described in detail in previous studies [4, 17]. Ovine prolactin (1 μg/mL) (NIH-PS13) was added as stated in the figure captions.

The kinase inhibitors staurosporine and GF 109203X were added 4 hr before prolactin, when the hormonal stimulus is short, and with prolactin for 24-hr stimulations.

At the end of the cultures, cells were saved and kept frozen until use.

Nb_2 Cell Cultures

Nb_2-11C cells, which are strictly dependent on prolactin to grow, were cultured under conditions described by Gertler *et al.* [5]. In brief, the cells were initially grown in fetal calf serum. They were then maintained for 24 hr in 10% prolactin-depleted horse serum. Ovine prolactin (NIH-PS13) was added at a concentration of 4 ng/mL to induce Nb_2 cell multiplication. Cells cultured in suspension were collected by centrifugation at different times as stated in the figure captions. The cells were rapidly cooled and kept frozen until use.

Different compounds were added to the culture medium to modulate prolactin action tentatively. Staurosporine (15 nM) and GF 109203X (1 μM) were added 4 hr before prolactin, when a short action of the hormone was examined. The elements were added with prolactin when cell growth over 24-hr periods was studied. TPA was added 10 min before the cells were collected.

Measurements of Protein Kinase Activities

Two dishes of 100-mm diameter were used for each point. Cells kept frozen at −80°C were homogenized in a buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, aprotinin, antipain, leupeptin, pepstatin A and chymostatin (5 μg/mL each), 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 50 mM β-glycerophosphate, 10 μM amonium molybdate and 1 mM Na_3VO_4. The high-speed centrifugation supernatant was used to measure total kinase activities. Alternatively, it was fractionated by using a Mono Q 5/5 column. The column was equilibrated with 20 mM Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, 0.5 mM EGTA and 0.2% NaN_3. The proteins were eluted by using a 40-mL linear concentration gradient of NaCl from 0 to 0.6 M at a flow rate of 1 mL/min. Fractions of 1 mL were collected and used for the determination of kinase activities.

CKII activity was measured by using the specific peptide substrate RRREEETEEE (0.5 μM) or partially dephosphorylated casein (2 mg/mL). The reaction mixture (total 50 μL) contained 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 50 μM [γ-^{32}P] ATP (≅500 cpm/pmol) and approximately 5-μ units of the enzyme. The reaction was started by addition of [γ-^{32}P] ATP. After 10 min of incubation at room temperature, the reaction was stopped by spotting 40 μL of the incubated product onto 5-cm^2 squares of P-81 phosphocellulose paper, which were then immersed in 1% H_3PO_4. After extensive washing, the filters were rinsed in ethanol and dried, and the bound radioactivity was estimated by liquid scintillation counting. Blanks were obtained when no substrate was added to the incubates. One CKII unit was defined as the amount of enzyme that catalyzed the incorporation of 1 μmol of ^{32}P per minute in the substrate. Under the standard conditions defined above, the phosphorylation of the substrates was linear for over at least 20 min following the addition of the enzyme. Protein concentrations were determined by using the Biorad assay.

MAP kinase activity was determined as follows. Crude cytosolic extracts or fractions from the Mono Q column (10 μL) were incubated in a total mixture of 50 μL containing 50 mM Tris-HCl, pH 7.5, MBP (0.5 mg/mL), 10 mM $MgCl_2$, 1.5 mM EGTA, 2 μM protein kinase A inhibitor peptide (Sigma), 10 μM calmidazolium and 100 μM [γ-^{32}P] ATP (≅1000 cpm/pmol). The incubations were carried out at room temperature for 10 min. The radioactivity incorporated in the substrate was determined as described for the measurement of CKII activity.

PKC activity was measured either by using the specific Gibco kit assay according to the manufacturer's recommendations or as described for MAP kinase, also by using MBP as substrate. Total and Ca^{2+}-independent PKC were discriminated by adding or not an excess of EGTA in the incubates.

All these methods have been described extensively and used in previous studies [21, 22].

Identification of MAP Kinase and PKC ξ by Immunoblotting

Fractions 9–11 and 13–16 were submitted to polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose filters. MAP kinase and PKC ξ were revealed in fractions 9–12 and 13–16, respectively, by using corresponding antibodies. Details of the method were described in a previous study [22].

Measurements of mRNA Concentration

Total RNA was extracted by using the acid phenol-chloroform-guanidinium isothiocyanate. RNA (10 μg) was separated in agarose gel and transferred to nylon membranes (Bioprobe). The different mRNAs were visualized by hybridizing 0.5 M sodium phosphate, 7% sodium dodecyl sulfate buffer with the corresponding ^{32}P-labeled cDNA probes. All these methods were described in previous studies [4, 17].

RESULTS

Effect of Prolactin on CKII

CKII was measured in mammary cell extracts after different times of prolactin action. Results shown in Fig. 1A indicate that prolactin did not alter CKII activity over a period of 24 hr.

CKII activity was measured in cytosol of Nb_2 cells at different times after addition of prolactin to the culture medium. CKII was stimulated by prolactin but only slowly. A slight increase in activity was visible after 12 hr of hormonal stimulation (Fig. 1B). Data from several independent experiments indicated that after 24 hr the activity was 2-fold higher than in the control when referred to cytosolic proteins (Fig. 1C).

Effect of Prolactin on MAP Kinase Activity

IN MAMMARY CELLS. Total MBP kinase activity was measured in cytosolic extracts at different times after the addition of prolactin. This activity was enhanced by prolactin after 10 min in rabbit mammary cells, and stimulation was maintained for 24 hr (not shown). To evaluate MAP kinase activity among the total MBP kinases, fractionation of cytosolic extracts was carried out by using Mono Q chromatography (Fig. 2A). Immunoblotting indicated that MAP kinases were in fractions 9–11 (Fig. 3A). After 10 min of prolactin action on mammary cells, MAP kinase activity was stimulated 2.5-fold (Figs. 2A, 4). This stimulation was obtained reproducibly with independent cultures. The stimulation of MAP kinase activity by prolactin was essentially transient and was no longer observed after 60 min or 24 hr (Fig. 5).

MAP kinases can be stimulated by phosphorylation, particularly when PKC is enhanced in cells. TPA, a potent stimulator of some of the lipid-dependent PKC, markedly stimulated mammary MAP kinase (Fig. 4). Staurosporine,

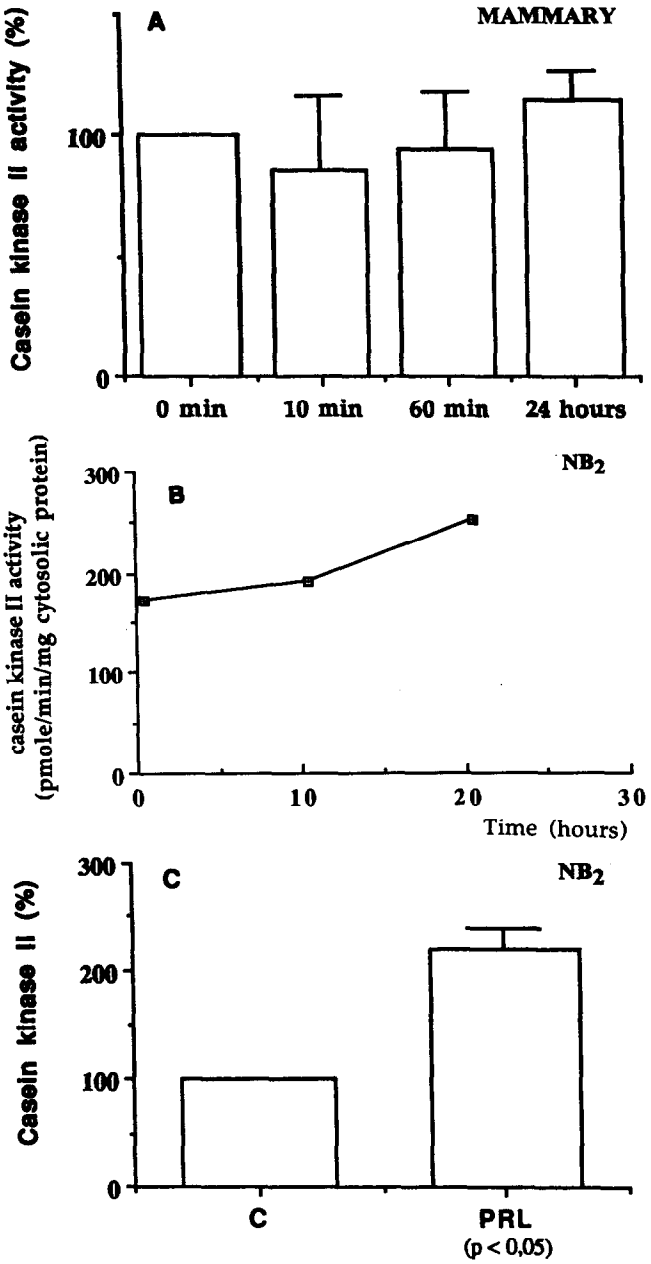

FIG. 1. Effect of prolactin on CKII activity in rabbit mammary and Nb_2 cells. The rabbit mammary cells were cultured on plastic support and treated with prolactin for 24 hr. CKII was measured in Mono Q eluates. Results are the means of percentages of CKII activity in pmol/min/cell ± SEM of duplicates from four independent cultures. (A) Rabbit mammary cells, (B) Nb_2 cells and (C) Nb_2 cells after 24 hr; 100% of CKII activity corresponded to 90 and 167 mol/min/mg cytosolic protein for mammary and Nb_2 cells, respectively.

an inhibitor of protein tyrosine kinase and PKC, did not alter mammary basal MAP kinase activity (not shown). When added with prolactin, this inhibitor surprisingly amplified the action of the hormone on MAP kinase activity (Fig. 2A). Unexpectedly, GF 109203X, a potent and specific inhibitor of PKC, stimulated mammary MAP kinase

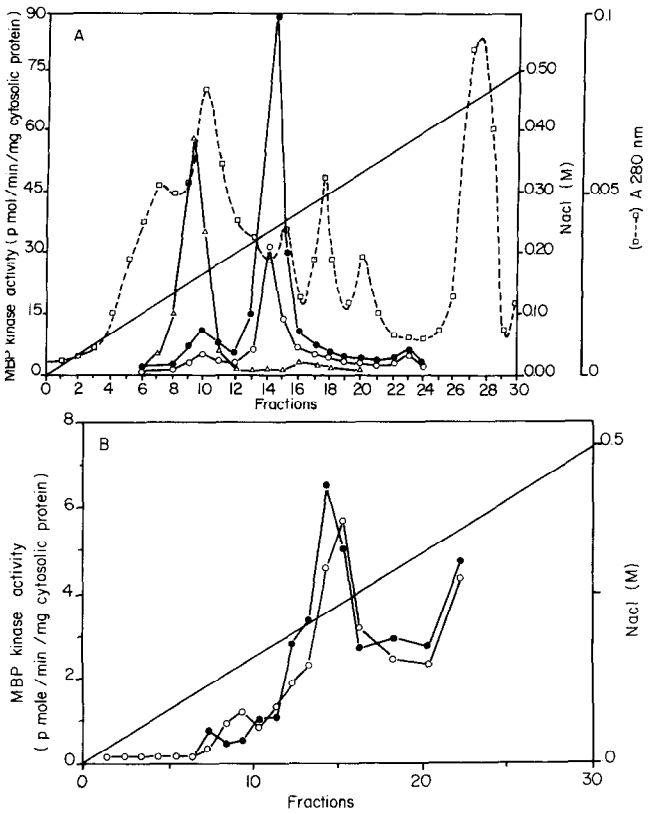

FIG. 2. Effect of PRL and St on different MBP kinase activities. The cytosolic extracts were fractionated on a Mono Q column, and MBP kinase activity was estimated in the different fractions. Cell extracts were prepared from control cells and from cells treated for 10 min with PRL. Fractions 9–11 contained MAP kinases, fractions 13–16 (see Fig. 3A, B) contained PKC and fractions 22–24 contained an unknown MBP kinase. (A) Rabbit mammary cell (open circle) control; (solid circle) + PRL; and (triangle) + PRL + St (15 nM) added 4 hr before PRL (square). Absorbance at 280 nm. (b) Nb_2 cells.

activity. This effect was not additive to the stimulation obtained with prolactin (Fig. 4).

IN Nb_2 CELLS. Prolactin was added to the Nb_2 cell culture medium for different periods of time ranging from 5 min to 24 hr. MAP kinase activity was estimated to the Mono Q column eluates of the cytosolic extracts. MAP kinase activity was identified in fractions 11 and 12 of the Mono Q eluates (Fig. 2B), and the presence of these kinases was revealed by immunoblotting (Fig. 3B). MAP kinase activity was low and essentially unaltered after 10 min (Fig. 2B) and at any time after addition of prolactin to the Nb_2 culture medium (not shown). These data show that the induction of Nb_2 cell multiplication by prolactin occurred without any significant stimulation of MAP kinase.

FIG. 3. Identification of MAP kinase in mammary and Nb_2 cell extracts. The presence of MAP kinase in pooled fractions 9–11 from the Mono Q columns (Fig. 2A, B) and revealed by Western blotting. The arrows indicate both forms of MAP kinase; (A) rabbit mammary cells, (B) Nb_2 cells.

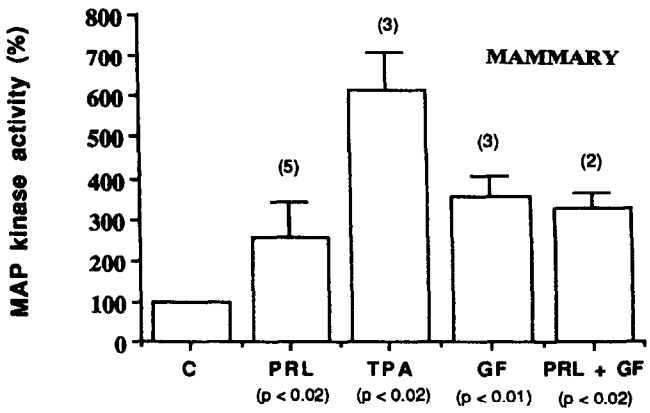

FIG. 4. Effect of PRL, TPA and GF on MAP kinase activity. The mammary cells were cultured on plastic support and stimulated by PRL for 10 min. TPA (60 μg/mL) was also added for 10 min. GF (1 μM) was added 4 hr before PRL. MAP kinase activity was measured in fractions 9–12 (Fig. 2A) by using MBP as a substrate. Results expressed as percentage of the control are the means ± SEM of several independent cultures. Numbers within parentheses indicate the number of experiments.

Effect of Prolactin on PKC Activity

IN MAMMARY CELLS. Prolactin markedly stimulated MBP kinase activity after 10 min (Fig. 2A, fractions 13–

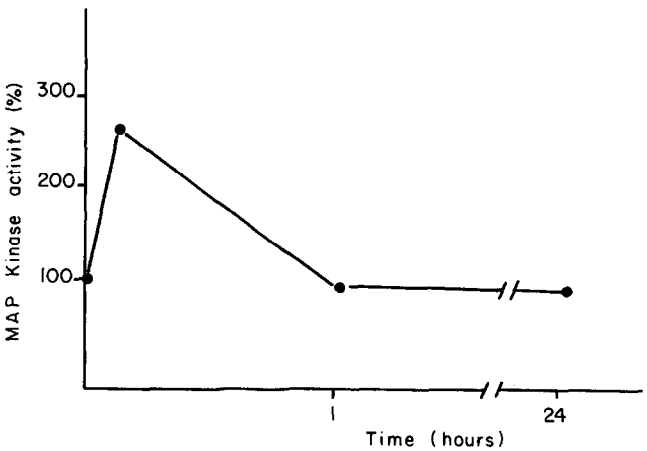

FIG. 5. MAP kinase induction in rabbit mammary cells under prolactin action. Primary cells cultured on plastic support were stimulated by PRL for different times and MAP kinase activity was evaluated in pooled fractions 9–11 from the Mono Q columns.

16). Several biochemical properties indicated that this protein kinase belongs to the PKC family. The protein kinase activity found in the 13–16 fractions was identified with the PKC kit assay. This kinase activity was inhibited by 2-AP (IC_{50} = 5 mM), 6-DMAP (IC_{50} = 0.5 mM), staurosporine (IC_{50} = 8 nM) and GF 109203X (IC_{50} = 0.02 μM). Staurosporine and GF 109203X did not completely inhibit MBP kinase activity. The residual activity may reflect the presence of PKC ξ, which is not inhibited by GF 109203X [23].

Different stimulators or modulators of PKC were tested to identify the protein kinase found in fractions 13–16 from the Mono Q column using MBP as substrate. This kinase activity was not modified by the presence of Ca^{2+}, was almost totally insensitive to phosphatidylserine and was somewhat stimulated by TPA. The kinase was significantly inhibited by polylysine and stimulated by poly [glu-tyr] 4:1.

The kinase was able to phosphorylate several substrates used by PKC. MBP, histone H1, GS peptide and protamine sulfate were good substrates, whereas histones H_2A, H-ILS, H-AP, casein and phosvitin were only weakly phosphorylated by the enzyme. Phosphatidylserine and TPA stimulated the kinase differently according to the substrate used.

To identify more precisely the PKC present in fractions 13–16 from the Mono Q column, several immunoblottings were done. Anti-PKC δ and ε antibodies gave no signal. Anti-PKC ξ antibody revealed the presence of a 71-KDa protein, which is a good candidate to be PKC ξ (Fig. 6A). The antibody also showed other bands corresponding to proteins smaller and larger than PKC ξ. These proteins may be other PKC. The anti-PKC ξ antibodies classically used are not completely specific and to some extent recognize other members of this family [24].

The effect of prolactin, GF 109203X and staurosporine on the Ca^{2+}-independent PKC found in fractions 13–16 from the Mono Q column was evaluated. Measurements were done after 10 min and 24 hr of prolactin action. The stimulatory effect of prolactin on PKC activity was strongly and only moderately inhibited by GF 109203X and staurosporine, respectively (Fig. 7).

Collagen I gel favors mammary cell differentiation and prolactin action on casein genes. The gel amplified the hormone effect on the Ca^{2+}-independent PKC. In three independent cultures, the PKC activity found in fractions 13–16 from the Mono Q was 2.84 ± 0.58-fold higher in cells cultured on collagen I gel and stimulated by prolactin than in control cells cultured on plastic support and stimulated by the hormone.

IN Nb_2 CELLS. Total and Ca^{2+}-independent PKC was evaluated in crude Nb_2 cytosolic extracts 24 hr after the addition of PRL into the medium of Nb_2 cells. Ca^{2+}-independent PKC represented 18% of total PKC activity (Fig. 8). PRL did not increase total PKC or Ca^{2+}-independent PKC. On the contrary, it decreased when referred to the concentration of cytosolic proteins (Fig. 8). During prolactin stimulation, total soluble proteins were greatly increased, and this action may have generated an apparent decrease in PKC activity under the hormonal action.

To evaluate PKC activity more precisely, the crude cytosolic extracts were fractionated by using Mono Q chromatography. The results presented in Fig. 2B indicate that an MBP kinase was stimulated by prolactin after 10 min (fractions 12–14). This effect was amplified after 24 hr (not shown).

Immunoblottings were carried out to tentatively identify the protein kinase in fractions 12–16. Data reported in Fig. 6B indicate that PKC ξ was present in fraction 15 from the Mono Q, as judged by the reaction with a specific antibody. The anti-PKC ξ antibody revealed not only a PKC at 71 KDa but also another molecule of 56 KDa of unknown nature, which was found in most cell extracts. PKC ξ is not stimulated by TPA [24] and is not inhibited by GF 109203X [23]. The PKC activity found in fraction 15 was essentially PKC ξ. The PKC activity found in tubes 13–14 and stimulated by prolactin could thus not be identified with certainty. It is one of the PKC isoforms that is Ca^{2+} independent and stimulated by phorbol esters [25].

Effect of PKC Inhibitors on Gene Induction by Prolactin

IN MAMMARY CELLS. Staurosporine and GF 109203X were added to the culture medium of rabbit mammary cells. Staurosporine very significantly reduced total Ca^{2+}-independent PKC activity after 2 hr (not shown). It was less efficient after 24 hr, possibly due to its partial degradation during the culture (Fig. 7). GF 109203X was a much better inhibitor (Fig. 7). The PKC activity shown in Fig. 7 represents only the Ca^{2+}-independent kinases, which are not all sensitive to staurosporine and GF 109203X. The total remaining PKC activity in cells was therefore very low. In a previous study staurosporine, which inhibits PKC and some tyrosine kinases, abrogated the induction of casein gene expression by prolactin [4]. Results shown in Fig. 9 reveal that GF 109203X did not alter the induction of αS-1-casein gene, although it did considerably reduce PKC activity (Fig. 7). In the test depicted in Fig. 7, PKC activity was measured in the presence of EGTA, essentially revealing Ca^{2+}-independent PKC. GF 109203X is a potent inhibitor of all PKC except for PKC ξ [23]. In the mammary cell, Ca^{2+}-dependent PKC are the most abundant [26, 27]. The residual PKC activity found after the addition of GF 109203X to the culture medium, possibly corresponding to PKC ξ, therefore represented a very small proportion of total cellular PKC (Fig. 7).

IN Nb_2 CELLS. Staurosporine and GF 109203X were added to Nb_2 cell culture medium with prolactin for 24 hr. As expected, prolactin induced Nb_2 cell multiplication. This effect was abolished by staurosporine but unaffected by GF 109203X. Staurosporine only partially reduced total PKC and did not reduce Ca^{2+}-independent PKC at all. On

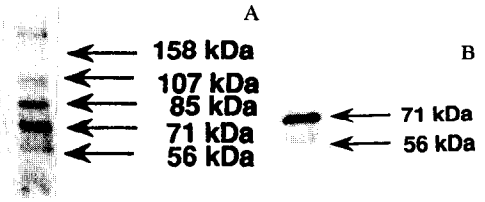

FIG. 6. Identification of PKC ξ in eluate from Mono Q columns. Aliquots from pooled fractions 15–16 were submitted to Western blotting with an anti-PKC ξ antibody. Arrows indicate protein molecular weight. (A) Rabbit mammary cell extract. (B) Nb_2 cell extract.

the contrary, GF 109203X almost completely abolished PKC activity (Fig. 8).

Previous studies have shown that the c-myc gene is rapidly induced [28] and that β-actin [29] stathmin [30] mRNAs are slowly induced by prolactin in Nb_2 cells. To evaluate the possible involvement of protein tyrosine kinase and PKC in these phenomena, staurosporine and GF 109203X were added with prolactin for 30 min and 24 hr.

As previously shown [28], prolactin induced c-myc mRNA accumulation after 30 min. This effect was attenuated by staurosporine but not by GF 109203X. Similarly, staurosporine, but not GF 109203X, reduced β-actin and stathmin mRNA accumulation (Fig. 10). The amplitude of the inhibition by staurosporine may have been underestimated by the fact that total RNA was multiplied by 6.5 after 24 hr of prolactin action and total cell number only by 2.

DISCUSSION

Results of the present study show that prolactin does not modify the activity of CKII in rabbit mammary cells. This type of kinase is involved in cell replication [8]. Under the experimental conditions of the present study, the mammary cells did not divide. Therefore, it is not surprising that CKII was not altered by prolactin. CKII is also able to phosphorylate and to stimulate a number of transcription factors [8]. In a recent study, MGF, the transcription factor induced by prolactin, was shown to be activated by CKII [31]. Results of the present study do not argue in favor of the idea that prolactin uses CKII to stimulate milk protein gene expression.

The slow stimulation of CKII by prolactin in Nb_2 cells suggests that this kinase is not specifically induced in prolactin action in these cells. This enhancement of CKII kinase II might rather reflect the high multiplication rate of the cells after 24 hr. This observation is in agreement with the role attributed to CKII in the multiplication cell cycle [32–34].

Prolactin consistently and transiently stimulated MAP kinase activity in the mammary cells. This kinase plays an important role in the early phase of the cell division cycle and activates several transcription factors [9]. It is not certain, however, that the induction of MAP kinase is an essential step in the mechanism of milk protein gene induction by prolactin. The hormone must be permanently present in the culture medium to maintain milk protein gene expression. Antiprolactin antibodies, colchicine and several protein kinase inhibitors that prevent induction of milk protein gene expression by prolactin rapidly deinduced these genes when added to the culture medium of mammary cells after prolactin [20, 35]. *In vivo*, but not in culture, prolactin is a potent growth factor for mammary cells. In culture, prolactin may participate in the induction of a multiplication cell cycle by stimulating MAP kinase activity and this stimulation may be abortive due to the lack of other growth factors in the culture medium.

Surprisingly, staurosporine did not prevent but rather amplified the stimulation of MAP kinase activity by prolactin. This kinase inhibitor prevents both the induction of casein gene expression by prolactin in rabbit mammary cells

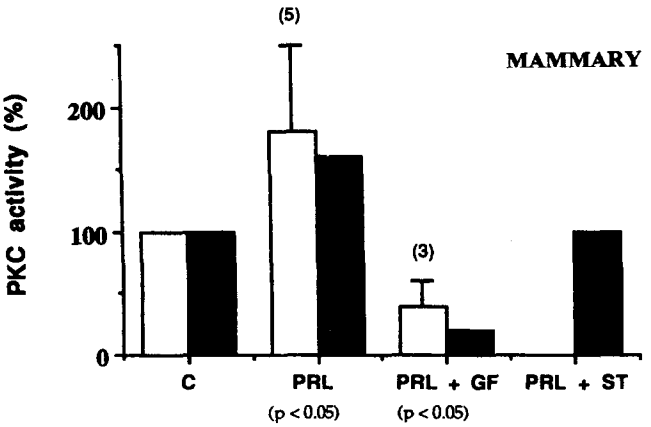

FIG. 7. Effect of PRL, GF and St on Ca^{2+}-independent PKC activity. PKC activity was measured in fractions 13–16 from the Mono Q column by using MBP as substrate in the presence of EGTA. GF 109203X (GF) (1 μM) and St 15 nM were added 4 hr before PRL. Results expressed as percentage of the control are the means ± SEM of several independent cultures. Numbers within parentheses indicate the number of experiments done with a stimulation of 10 min (open bar); only one measurement was done after 24 hr (solid bar).

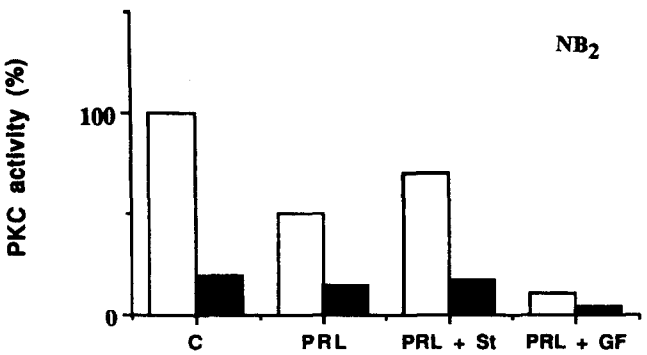

FIG. 8. Effect of PRL, GF and St on total and Ca^{2+}-independent PKC in Nb_2 cells. The agents were added to Nb_2 cell culture medium for 24 hr. Total and Ca^{2+}-independent PKC were measured in crude cell extract by using MAP as a substrate (open bar) for total PKC; solid bar indicates Ca^{2+}-independent PKC.

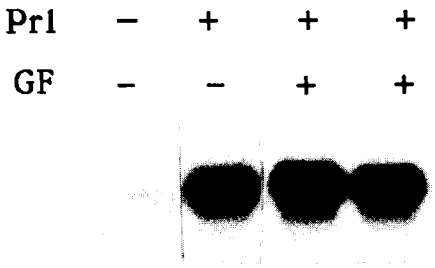

FIG. 9. Effect of GF 109203X on the induction of αSI-casein mRNA accumulation by PRL. PRL and GF (1 μM) were added for 24 hr to mammary cells cultured on plastic support. The concentration of the mRNA was estimated by Northern blotting by using the corresponding labeled cDNA probes. In all cases, 100 μg of total RNA were used.

[4] and MGF activation by inhibiting JAK_2 [19, 20], a protein tyrosine kinase stimulated by prolactin [12, 19]. The fact that staurosporine and GF 109203X did not prevent MAP kinase activation by prolactin strongly suggests that this activation occurred independently of PKC and JAK_2 kinase activation by the hormone. Therefore, prolactin seems to deliver several messages simultaneously in the mammary cells by different transduction mechanisms.

The fact that MAP kinase activity was not at all enhanced by prolactin in Nb_2 cells was quite unexpected. A rapid marked and transient stimulation of MAP kinase activity is observed in quiescent cells after addition of growth factors [9, 36]. This observation casts some doubt as to whether an increase in MAP kinase activity is a prerequisite for the triggering of cell multiplication. Several recent studies done with different cell types have led to a similar conclusion [37, 38]. It is generally admitted that cell division induced by different growth factors involves the activation of a cascade including ras, raf 1 and MAP kinase [9, 36]. In a recent work, prolactin was shown to induce raf 1 kinase [39]. In another study, Buckley *et al.* [40] reported that prolactin rapidly phosphorylates MAP kinase in Nb_2 cells; however, whether this phosphorylation triggered an increase in MAP kinase activity was not demonstrated. According to our results, the activation of raf 1 kinase and the phosphorylation of MAP kinases are not followed by a stimulation of this enzyme. Similar observations were made in several other systems [41, 42], suggesting that the kinase cascades leading to the initiation of cell multiplication might be more complex and diversified than initially imagined. In a recent work, a transcription factor similar to p91 and activated by interferon γ, was to be activated by prolactin in Nb_2 cells [14, 16, 20]. In Nb_2 cells, the essential part of the multiplication signal delivered by prolactin may be transmitted to the nucleus via the activation of a protein of the stat family such as p91.

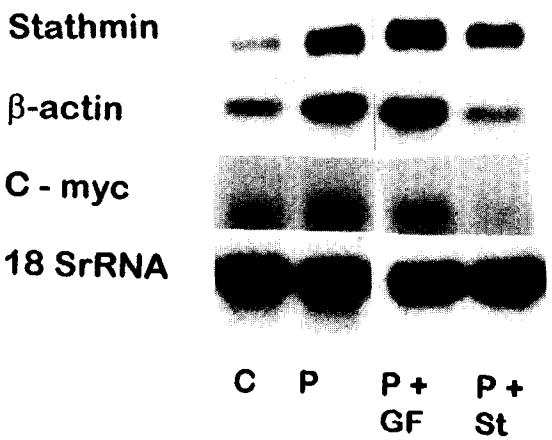

FIG. 10. Effect of St and GF on the induction of stathmin, β-actin and c-myc mRNA accumulation by prolactin in Nb_2 cells. Prolactin was added for 30 min to induce c-myc and for 24 hr to induce β-actin and stathmin genes, respectively. St (15 nM) and GF (1 μM) were added. The presence of the mRNAs was revealed by using corresponding labeled cDNA probes. In all cases, 10 μg of total RNA were used.

Prolactin also stimulated at least one Ca^{2+}-independent PKC in Nb_2 cells. This PKC was not completely identified in the present study, but the fact that it was inhibited to a large extent by GF 109203X suggests that it is not only the PKC ξ isoform.

The activation of milk protein genes was shown to be blocked by staurosporine [4]. This compound may act by inhibiting PKC or a protein tyrosine kinase such as JAK_2. The fact that GF 109203X did not prevent αS1-casein mRNA accumulation at all suggests that staurosporine acts by inhibiting JAK_2 or another kinase and that PKC is not strictly required for prolactin to induce milk protein gene expression. In a recent study, PKC was shown to activate MGF in a cell-free system [43]. Results of the present study suggest that PKC action on MGF in this cell-free system might not reflect a natural event. MGF might thus be activated in a cell-free system by PKC but not by nonphysiological phosphorylation.

The role of the PKC activated by prolactin is not known. Prolactin exerts several actions in the mammary cells. Among these is the stimulation of milk secretion [44]. The fact that prolactin stimulates PKC more intensively when cells are cultured on collagen gives additional support to the idea that prolactin might contribute to mammary cell differentiation by transduction mechanisms independent of that implied in milk protein gene activation.

Prolactin did not greatly modify total and Ca^{2+}-independent PKC in Nb_2 cells. At least one Ca^{2+}-independent PKC was stimulated by prolactin after 10 min and after 24 hr. The exact nature and role of this PKC were not determined in the present work.

In recent studies [5, 7], PKC was shown to favor prolactin action on Nb_2 cell multiplication. In the present study, two protein kinase inhibitors were used. Staurosporine inhibits Ca^{2+}-dependent PKC and some tyrosine kinases. This inhibitor blocks JAK_2 activity, which plays an essential role in the transmission of the prolactin message into Nb_2 [10, 12, 14] and mammary cells [12, 19]. GF 109203X inhibits all PKC at the concentration of 1 μM, except for PKC ξ

TABLE 1. Comparison of the effects of PRL and protein kinase stimulators and inhibitors in Nb_2 and mammary cells

	Agent	Nb2 cells	Mammary cells
MAP kinase	PRL	—	↗
PKC (fractions 13–14)	PRL	↗	—
PKC (fractions 15–16)	PRL	—	↗
CKII	PRL	↗	—
MAP kinase	TPA	—	↗
PKC (fractions 13–14)	TPA	↗	↗
MAP kinase	St	—	—
MAP kinase	St + PRL	—	↗
MAP kinase	GF	—	↗
MAP kinase	GF + PRL	—	↗
Cell multiplication	PRL	↗	—
Cell multiplication	PRL + St	—	—
Cell multiplication	PRL + GF	↗	—
Casein gene expression	PRL	—	↗
Casein gene expression	PRL + St	—	—
Casein gene expression	PRL + GF	—	↗

[23]. The fact that staurosporine, but not GF 109203X, prevented prolactin action on Nb_2 cell multiplication and on c-myc and stathmin mRNA accumulation strongly suggests that a tyrosine kinase, possibly JAK_2, plays the most crucial role in the hormonal effects. The fact that prolactin induces tyrosine kinase activity and tyrosine phosphorylation in Nb_2 cells also supports this view [45, 46]. Therefore, PKCs seem to be only marginally involved in Nb_2 cell multiplication, a fact in agreement with data reported by Meyer *et al.* [47].

PKC does not seem essential for prolactin action in rabbit primary mammary cells or in mouse HC11 mammary cells [48]. Prolactin stimulates PKC in several other cellular systems [49–51]. The real importance of this phenomenon has, however, not been completely evaluated.

A systematic comparison of the prolactin effect in Nb_2 and mammary cells revealed that the hormone does not have the same mechanism of action in the two cell types (Table 1). This finding may of course reflect the fact that prolactin stimulates Nb_2 cell multiplication but not differentiation, whereas it induces differentiation and milk protein gene expression but not multiplication in mammary cells.

We thank E. Devinoy, A. Sobel, M. Piechaezyk, M. Buckingham and J.P. Bachelerie for providing cDNAs for rabbit αS1-casein, human stathmin, human c-myc, mouse β-actin and 18SrRNA. We also thank Dr. O. Bensaude and Dr. M.F. Dubois for their advice on the performing of immunoblottings.

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