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Original article

Protein kinase C is not necessary for β-casein gene induction by prolactin in HC11 mouse mammary cells

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Summary — HC11 mouse mammary cells cultured in the presence of insulin, cortisol and prolactin for 24 h accumulated β-casein mRNA. When the specific inhibitor of protein kinase C, GF 109203 X, was added to the medium with the hormones, the accumulation of β-casein mRNA was unaltered, although the protein kinase C activity was almost completely suppressed. This suggests that protein kinase C is not strictly necessary for prolactin to induce milk protein gene expression.

HC11 cells / β-casein / prolactin / protein kinase C / GF 109203 X

Résumé — La protéine kinase C n'est pas nécessaire pour l'induction des gènes de la caséine-β par la prolactine dans les cellules mammaires de souris HC11. *Les cellules mammaires de souris HC11 cultivées en présence d'insuline, de cortisol et de prolactine accumulent l'ARNm de la caséine-β. En présence de l'inhibiteur de la protéine kinase C, le GF 109203 X, l'accumulation de l'ARNm de la caséine-β a encore lieu bien que la protéine kinase C soit presque totalement inhibée. Ceci suggère que la protéine kinase C n'est pas strictement nécessaire pour que la prolactine induise l'expression des gènes des protéines du lait.*

cellules HC11 / caséine β / prolactine / protéine kinase C / GF 109203 X

INTRODUCTION

Protein kinase C is present in the mammary gland at varying concentrations. The highest levels are found during pregnancy when the development of the mammary gland occurs; these levels are reduced during lactation (Caufield and Bolander, 1986; Lavandero *et al*, 1992). This suggests that this type of kinase is more necessary for mammary cell multiplication than for differentiation.

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In research carried out several years ago, it was shown that the activators of protein kinase C, phorbol esters, could not mimic prolactin action and induce milk protein gene expression. In contrast, these compounds inhibited the prolactin effect (Martel *et al,* 1983). More recently, it was shown that several protein kinase C inhibitors, H_7 (1-(5-isoquinolinolylsulfonyl)-2-methylpiperazine), polymixin B, and sphingosin did not prevent prolactin from inducing casein synthesis in cultured rabbit mammary cells (Bayat-Sarmadi and Houdebine, 1993). However, 2 other protein kinase C inhibitors, staurosporine and quercetin, totally cancelled the prolactin effect (Bayat-Sarmadi and Houdebine, 1993). The 2 compounds are known to inhibit several types of kinases, particularly tyrosine kinases. These data are not therefore contradictory. In a more recent study, we observed that GF 109203 X (Toullec *et al,* 1991) which is also known as Gö 6850 (Martiny-Baron *et al,* 1993) and which is a potent specific inhibitor of most of the protein kinase C, did not prevent prolactin from inducing casein synthesis in rabbit primary mammary cells (Mitev *et al,* unpublished data). From these experiments, it was deduced that protein kinase C is probably not strictly involved in the transduction mechanism of the prolactin message to milk protein genes.

In a recent publication, Marte *et al* (1994) revealed that a derivative of staurosporine, CGP 41251, prevented prolactin from inducing β-casein gene expression in the mouse mammary cell line HC11 (in the presence of insulin and cortisol). This compound was previously shown to act preferentially on protein kinase C, although the authors of that study did not exclude that it may inhibit various other protein kinases (Meyer *et al,* 1989). From their study, Marte *et al* (1994) concluded that protein kinase C is involved in the action of prolactin on milk protein genes.

HC11 mouse mammary cells and primary rabbit mammary cells are quite different and it cannot be excluded that protein kinase C plays a different role for the induction of casein synthesis by prolactin in these 2 cell types.

In order to clarify this point, the induction of β-casein gene expression by lactogenic hormones in HC11 was carried out in the presence and the absence of GF 109203 X. Protein kinase C activity was also measured in cell extracts at the end of the culture to evaluate the inhibitory effect of GF 109203 X.

MATERIALS AND METHODS

HC11 cells were kindly supplied by Dr B Groner. They were cultured under previously defined conditions (Ball *et al,* 1988). Cells were cultured to hyperconfluence in the presence of EGF (10 ng/ml). This growth factor was then withdrawn and the lactogenic hormones insulin (5 μg/ml), cortisol (500 ng/ml) and ovine prolactin (5 μg/ml) were added to the culture medium to induce milk-protein gene expression. GF 109203 X, H-7, calphostin C and staurosporine were also added at concentrations of 1 μM, 50 μM, 1 μM and 25 nM, respectively, with the hormones as stated in legends of the figures. One day later, the cultures were stopped and the cells were kept frozen at −20°C until use.

All the cells used for the measurements of protein kinase C activity and β-casein mRNA concentration were cultured simultaneously. Different dishes were used for the extraction of total proteins and RNA.

β-Casein mRNA was measured by dot and Northern blotting as previously described (Puissant *et al,* 1994) using mouse β-casein cDNA as a probe (Yoshimura *et al,* 1986) (kindly provided by Dr T Oka). The specific activity of the probe was 10^9 cpm/μg of DNA. The probe was added to the hybridization medium at the concentration of 3×10^6 cpm/ml. The probe hybridized to the pieces of membrane was estimated by scintillation counting in the case of dot blotting and by a 4 d autoradiography for Northern blots.

Protein kinase C in cell extracts was measured as follows. The total protein kinase C activity was determined according to the protocol defined in the Gibco assay kits. Ca^{2+}-indepen-

dent protein kinase C activity was determined in the presence of 5 μM ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA).

RESULTS

After 1 d of culture in the presence of the protein kinase inhibitors, the protein kinase C activity in cell extracts was considerably reduced. GF 109203 X appeared to be the most potent compound and no more than 10% of the total protein kinase C activity remained in the extracts (fig 1).

A test to evaluate the Ca^{2+}-dependent and Ca^{2+}-independent enzymes revealed that most of the protein kinase C activity present in HC11 is Ca^{2+}-dependent (fig 1). This is in agreement with the work of Marte *et al* (1994) in which it was shown that the α-isoform of protein kinase C is the only member of the Ca^{2+}-dependent family in these cells. In the rat mammary gland, the α-isoform of protein kinase C has also been shown to be the dominant activity (Connor and Clegg, 1993). GF 109203 X is known to inhibit all the Ca^{2+}-dependent and, to some extent several of the Ca^{2+}-independent protein kinases C. The fact that it inhibited almost all the protein kinase C activity in HC11 (Toullec *et al*, 1991; Martiny-Baron *et al*, 1993) is therefore not surprising.

Dot blot analysis indicated that the β-casein mRNA accumulated in HC11 after 24 h in the presence of the lactogenic hormones (fig 2). This hormonal effect was completely suppressed by staurosporine, and unaltered or slightly amplified by GF 109203 X, H-7 and calphostin C. This point was confirmed by Northern blot analysis (fig 3). None of the compounds showed any sig-

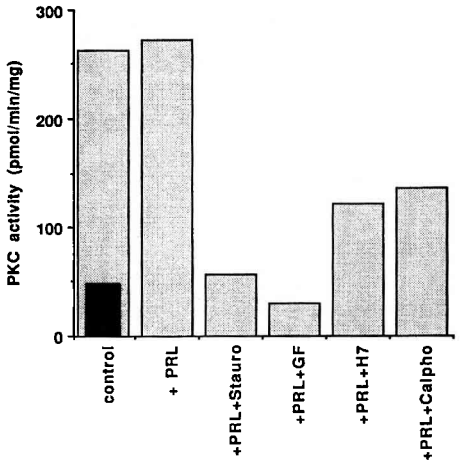

Fig 1. Protein kinase C (PKC) activity in HC11 cell extracts. Cells at hyperconfluency were cultured for 24 h in the presence in all cases of insulin (5 μg/ml) and cortisol (500 ng/ml). Prolactin (PRL) (5 μg/ml), H-7 (50 μM), GF 109203 X (1 μM), calphostin C (calpho) (1 μM) and staurosporine (25 nM) were added as stated in the figure. In the control, the medium was supplemented with insulin and cortisol only. Total protein kinase C was estimated as depicted in *Materials and methods*. In the control, the Ca^{2+}-independent activity was also evaluated (■). The results are the means of duplicates from 2 independent dishes.

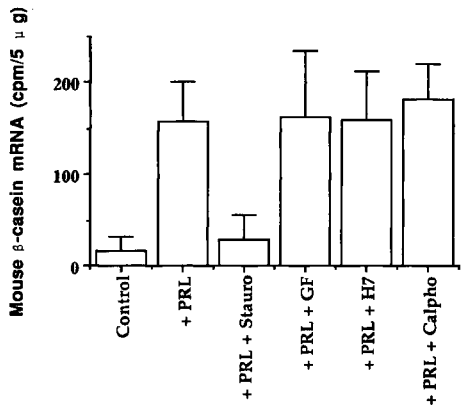

Fig 2. β-Casein mRNA measurements by dot blotting. Total RNA (5 μg) was spotted on 1 cm^2 nylon membrane (Biophylon Z+ Bioprobe). The loaded membranes were hybridized with [^{32}P]-labelled β-casein cDNA for 18 h. The results are the means ± SEM of triplicates from 4 independent dishes. See figure 1 for definition of the cultures.

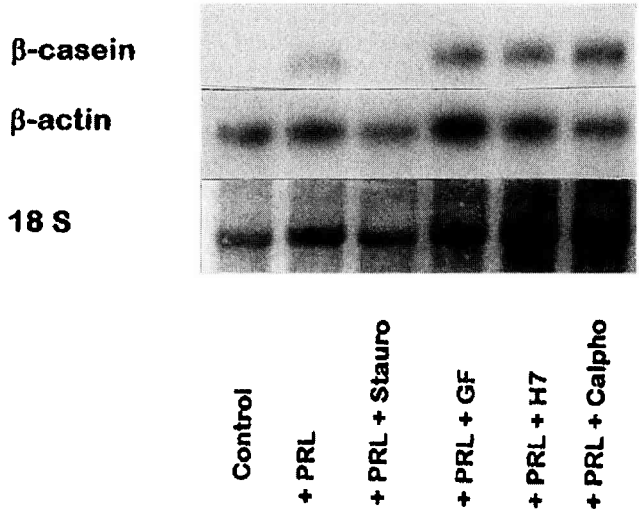

Fig 3. Northern blot analysis of β-casein mRNA. Total RNA (20 μg) were separated on agarose, transferred to nylon membrane (Biohylon Z+, Bioprobe) and hybridized with [^{32}P]-labelled β-casein cDNA. Each fraction is a mixture of 25 μg of RNA from each dish corresponding to a given treatment (see figure 1 for definition). The membranes was rehybridized with a labelled β-actin probe. The concentration of 18 S rRNA visible in UV light on the nylon membrane after the blotting was also taken as a measure of the concentration of RNA loaded in each well.

nificant cytotoxic effect as judged by the presence of β-actin mRNA. This agrees with our previous study carried out with rabbit primary mammary cells (Bayat-Sarmadi and Houdebine, 1993).

The apparent slightly lower concentration of β-actin mRNA in lanes 1, 2 and 3 of figure 2 is due to the fact that slightly lower amounts of total RNA were present in the lanes. This is revealed by illuminating the gel with UV light at the end of the electrophoresis and blotting. The dot blot analysis of figure 3 also supports this view. A low cytotoxic effect cannot be totally ruled out for staurosporine, but does not seem to account for its strong inhibitory effect on β-casein mRNA accumulation.

DISCUSSION

The results of this work show unambiguously that several protein kinase C inhibitors considerably reduce the enzyme activity when added to the culture medium of HC11 cells. Only staurosporine cancelled the induction of β-casein gene expression. This drug is known to be a potent inhibitor of several tyrosine kinases and interrupts the transduction mechanisms of cytokines that involve the activation of Jak and Tyk tyrosine kinases (Kishimoto *et al*, 1994). Staurosporine probably acts in the mammary cells by inhibiting the Jak 2 kinase which has recently been shown to be involved in the prolactin transduction mechanisms (Campbell *et al*, 1994; Dusanter-Fourt *et al*, 1994; Gouilleux *et al*, 1994; Rui *et al*, 1994). The fact that GF 109203 X, H-7 and calphostin C did not prevent the induction of β-casein gene transcription strongly suggests that a protein kinase C is not strictly involved in the prolactin transduction mechanism. This conclusion is also supported by the fact that in the primary rabbit mammary cell, the rapid activation of mammary gland factor (MGF) by prolactin, a transcription factor which plays a major role in milk protein gene expression (Gouilleux *et al*, 1994), is cancelled by staurosporine but not by H-7 and GF 109203 X (N Tourkine *et al*, unpublished data). The discrepancy between the present

work and that published by Marte *et al* (1994), who concluded that protein kinase C is involved in prolactin action on milk protein genes, might be explained by the fact that the staurosporine derivative CGP 41251 retained the capacity to inhibit tyrosine kinase. A direct measurement of Jak 2 activity under CGP 41251 action could answer the question of whether this compound inhibits some tyrosine kinases in addition to protein kinase C.

A low activity of protein kinase C was still detected after the action of GF 109203 X. It is not certain that all this remaining activity was protein kinase C. Indeed, a residual kinase activity corresponding to about 5% of the control without the inhibition was still measurable in the absence of the protein kinase C substrate. It cannot be completely excluded that a minor protein kinase C isoform remained active despite the presence of GF 109203 X. The involvement of a protein kinase C in prolactin action on casein genes is very unlikely.

The conclusion of the present work does not exclude that protein kinases C are involved in some of the prolactin actions. Indeed, protein kinase C seems necessary for prolactin to induce Nb2 cell proliferation (Gertler *et al,* 1985; Rillema *et al,* 1989), although this fact has recently been questioned (Meyer *et al,* 1992). Protein kinase C also seems to be required for the activation of ornithine decarboxylase by prolactin in Nb2 and mammary cells (Waters *et al,* 1989). Prolactin action in astrocytes (DeVito *et al,* 1991), hypothalamus (DeVito *et al,* 1993) and smooth muscle cells (Sauro and Zorn, 1991) seems to require protein kinase C activity.

In the rabbit mammary gland, protein kinase C might favour milk secretion (Ollivier-Bouquet and Aubourg, 1992) and a recent study has also indicated that prolactin stimulates a Ca^{2+}-independent protein kinase C (V Mitev *et al,* unpublished data).

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