5-Hydroxyeicosatetraenoic Acid and Phorbol Ester Stimulate Prolactin Release from Rat Anterior Pituitary Cells by Different Mechanisms

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Abstract

The relationship between 5-hydroxyeicosatetraenoic acid (5-HETE) and calcium-activated, phospholipid-dependent protein kinase (protein kinase C) in prolactin (PRL) release was investigated in rat anterior pituitary cells. Arachidonic acid or 5-HETE, a 5-lipoxygenase metabolite of arachidonic acid, is known to cause a significant concentration-dependent increase in PRL release. Phorbol 12-myristate 13-acetate (PMA) and dioctanoylglycerol (diC8) have also been known to stimulate PRL release from pituitary cells, so we showed that these PRL releases were correlated with the activation of protein kinase C, that is, they induced dose-dependent translocation of protein kinase C from the cytosol to the membrane. Arachidonic acid, however, did not cause a significant change in the distribution of protein kinase C. We also showed that the PRL release induced by arachidonic acid and that induced by 5-HETE were additional to that by 100 nM PMA.

Thus we suggested that the signals for the stimulation of PRL release sent by arachidonic acid and 5-HETE would be different from the signal sent through protein kinase C by PMA.

Thyrotropin-releasing hormone (TRH) is thought to release PRL through a calcium-dependent process in pituitary cells (Rebechi et al., 1983; Drust and Martin, 1982). Phospholipid hydrolysis and calcium influx have been observed as early events after

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TRH binds to its receptor (Rebechi et al., 1983; Drust and Martin, 1982). Recently, diacylglycerol formed during phospholipid hydrolysis was shown to be a potent activator of calcium and phospholipid-dependent protein kinase (protein kinase C) (Kikkawa et al., 1982; Nishizuka, 1984; Turgeon et al., 1984). We previously found that protein kinase C was a mediator of gonadotropin releasing hormone (GnRH) in gonadotrophs and that GnRH caused translocation of protein kinase C activity from
the cytosol to the membrane (Catt et al., 1985). This translocation of protein kinase C was followed by a time- and dose-dependent release of LH. PMA is known to be a direct activator of protein kinase C, and also stimulates LH release from pituitary cells (Hirota et al., 1986). This suggests a significant role of protein kinase C in the release of PRL.

On the other hand, TRH induced arachidonic acid release and the exogenous addition of arachidonic acid also stimulated PRL release from pituitary cells (Canonico et al., 1983; 1985). Arachidonic acid was released by phospholipase A$_2$ which was activated by calcium (Canonico et al., 1985; Grandison, 1984). Moreover, 5-hydroxyeicosatetraenoic acid (5-HETE) which was a 5-lipoxygenase product of arachidonic acid was recently proposed to be a potential mediator of the action of TRH (Koike et al., 1984).

In this work we examined the relative contributions of 5-HETE and protein kinase C by analyzing PRL release and protein kinase C activity.

**Materials and Methods**

**Drugs**

5-HETE (a gift of Ono Pharmaceutical Co., Ltd., Osaka, Japan), arachidonic acid (Funakoshi Pharmaceutical Co., Ltd., Tokyo, Japan) and phorbol 12-myristate 13-acetate, dioctanoylglycerol (Sigma, St. Louis, MO) were dissolved in 100% dimethylsulfoxide (DMSO) and then diluted to the desired concentration with RPMI medium (Handai Biken, Osaka, Japan). The maximum concentration of DMSO in the culture medium was 0.25%, and this concentration did not affect PRL release by control cells.

**Cell culture**

Female Wistar rats (200–250 g) were decapitated in the morning, and their anterior pituitary glands were quickly removed and placed in complete medium (RPMI containing 2.5% fetal calf serum, 7.5% horse serum, 100 μg/ml gentamicin sulfate, 50 μg/ml streptomycin sulfate, and 125 μg/ml penicillin G). The neurointermediate lobes were removed, and the anterior lobes were cut into 1 mm$^3$ pieces with a scalpel. The tissue fragments were exposed to trypsin (0.25% ; Handai Biken) for 25 min at 37°C, and centrifuged (200×g ; 5 min). Then they were exposed to pancreatin (25 μg/ml ; Grand Island Biological Co., Grand Island, NY) for 15 min at 37°C, centrifuged (200×g ; 5 min), and resuspended in Minimal Essential Medium (Handai Biken). The tissue blocks were disrupted by pipetting in plastic pipettes with tapering tips until a single cell suspension was obtained. Then the cells were centrifuged and washed to remove extracellular trypsin and pancreatin. They were then suspended in a small volume of complete medium and counted, and their viability was examined by the trypan blue exclusion test. They were seeded into Falcon 24-well plates (Falcon, Oxnard, Ca) at density of $0.5 \times 10^6$ viable cells/well and incubated at 37°C for at least 4 days in a humidified atmosphere of 5% CO$_2$-95% air to allow them to become attached to the cells. On the day of the experiment, they were washed twice with serum-free RPMI medium containing antibiotics and then incubated for 2 h with various concentrations of test substances or vehicle. At the end of the incubation, the medium was quickly removed and stored at −20°C for PRL assay.

**RIA**

PRL was determined by double antibody RIA using material and protocols supplied by the NIADDK of the National Institutes of Health (Bethesda, MD). All samples were assayed in duplicate, and the results expressed in terms of standard PRL-RP-2 (intraassay variability, <6%; interassay variability, <10%) (Koike et al., 1984).

**Protein kinase assay**

Protein kinase C was assayed by measuring calcium- and phospholipid-dependent phosphorylation of H1 histone in the presence of diolein (Hirota et al., 1986; Takai et al., 1979). The assay mixture (0.25 ml) contained 20 mM Tris-HCl buffer (pH 7.5), 1.25 μmol of magnesium nitrate, 50 μg of histone, 0.2 μg of 1, 2-diolein, 5 μg of phosphatidylycerine, 0.125 μmol of CaCl$_2$, 2.5 nmol of $[^{32}P]$ ATP (5–10×10$^4$ cpm/nmol) and 30–50 μl of the sample. Basal activity was measured in the presence of
125 nmol of EGTA instead of 1, 2-diolein, phosphatidylserine, CaCl₂. After incubation for 4 min at 30°C, the phosphorylated histones were precipitated by adding 25% trichloroacetic acid, and washed on membrane filters (pore size 0.45 μM, Millipore Corp.) and radioactivity was measured in an Isocap 300 liquid scintillation counter.

**Results**

*Effect of PMA and arachidonic acid on protein kinase C activity and PRL release*

Fig. 1. shows effects of various concentrations of PMA on protein kinase C activity in the cytosol and membrane...
fractions. The maximum decrease in the activity in the cytosol, and the maximum increase in the membrane-associated protein kinase C activity were observed after treatment with 100 nM PMA, a concentration that caused maximal stimulation of PRL release from pituitary cells. The time courses PRL release and change in protein kinase C activity after stimulation with 100 nM PMA are shown in Fig. 2. Translocation of protein kinase C from the cytosol to the membrane fraction was almost complete after PMA stimulation for 10 min, while PRL release stimulated by PMA increased time dependently. PMA-stimulated PRL release was evident within 10 min.

Fig. 3. Concentration dependence of diC8-stimulated PRL release (above) and redistribution of protein kinase C activity between the cytosolic and membrane fractions (below). Triplicate cultures of pituitary cells (2×10⁶) were treated with the indicated concentrations of diC8 for 1 h at 37°C. The PRL in aliquots of the culture medium, and the protein kinase activities of the cytosol (●) and membrane (○) fractions of cells were measured. Values are the means±SEM for three determinations.

Fig. 4. Concentration dependence of arachidonic acid stimulated PRL release (above) and redistribution of protein kinase C activity between the cytosolic and membrane fractions (below). Triplicate cultures of pituitary cells (2×10⁶) were treated with the indicated concentrations of arachidonic acid for 2 h at 37°C. The PRL in aliquots of the culture medium, and protein kinase activities of cytosol (●) and membrane (○) fractions of cells were measured. Values are the means±SEM for three determinations.
10 min, and consistent with the rapid activation and redistribution of protein kinase C activity.

Dioctanoylglycerol (diC8), which is the analog of 1, 2-diacylglycerol and has the ability to activate protein kinase C (Lapetina et al. 1985). diC8 decreased the cytosol protein kinase C activity and increased the membrane-associated activity in a dose dependent manner. The release of PRL from pituitary cells was increased in the same manner (Fig. 3). The maximum effects were observed after treatment with 20 µM diC8, which was the same concentration that was shown to activate protein kinase C in human platelets (Lapetina et al., 1985).

Arachidonic acid also stimulated PRL release in a dose dependent manner, but caused no detectable change in the distribution of cytosolic and membrane-associated protein kinase C at concentrations of up to 10^-4 M (Fig. 4).

**Effects of arachidonic acid and 5-HETE on PMA induced PRL release**

Arachidonic acid and 5-HETE, which is a metabolite of arachidonic acid formed by 5-lipoxygenase, both stimulated PRL release from anterior pituitary cells concentration-dependently (Fig. 5, 6). PRL release was increased 5-fold by 10 µM arachidonic acid (Fig. 5), and 3.7-fold by 50 µM 5-HETE (Fig. 6). Arachidonic acid had an additive effect with 100 nM PMA in increasing PRL and its effect was concentration dependent (Fig. 5). Similarly, 5-HETE also increased PMA-induced PRL release in the same manner (Fig. 6).

At the concentrations tested, arachidonic acid and 5-HETE did not affect the viability of pituitary cells stained with Trypan Blue.

![Fig. 5. Effect of PMA on AA induced PRL release.](image-url) Cells were incubated 2 h with increasing concentrations of AA (1–50 µM) or PMA (100 nM) + AA (1–50 µM). PMA + AA (10–50 µM) stimulated PRL release significantly (p<0.001). Values are the means±SEM for four determinations.
Discussion

Recent studies have suggested that the interaction of TRH with its receptor in pituitary cells induces phosphatidyl inositol turnover and Ca\(^{2+}\) influx. The diacylglycerol formed during phosphatidyl inositol breakdown is a potent activator of protein kinase C. The relative contributions and interactions of protein kinase C and arachidonic acid in the control of PRL release are still unknown. Phospholipase A\(_2\) activated by Ca\(^{2+}\) cleaves arachidonic acid from phosphatidylcholine, phosphatidyl-inositol and phosphatidylethanolamine correlated with release of PRL (Canocico et al., 1983, 1985). A concentration-dependent release of PRL was observed following the exogenous addition of arachidonic acid (Fig. 5). Furthermore, 5-HETE, a product formed by 5-lipoxygenase, also stimulated PRL release (Fig. 6), and the release of other pituitary hormones (Miyake et al., 1988). In some endocrine cells, 5-HETE or other metabolites, such as leukotriene A4 or B4, may have a more potent stimulatory effect than either 5-HETE or 5-HPETE (Naccache et al., 1982). However, 12- and 15-hydroxyeicosatetraenoic acid-like products were found to not stimulate hormone release from the rat anterior pituitary (Naor et al., 1985).

Several factors that cause the release of pituitary hormone have been demonstrated to increase the hydrolysis of pituitary phospholipid (Berridge, 1981). Phospholipase C-induced phospholipid hydrolysis increases the intracellular levels of free diacylglycerol (Nishizuka, 1984), which may in turn activate protein kinase C present in the anterior pituitary (Niedel et al., 1983). Moreover, PMA, a potent protein kinase C activator (Castagna et al., 1982), has been found to stimulate the secretion of LH, TSH, GH and PRL (Hirota et al., 1986; Judd et al., 1986; Osborne and Tashjian, 1981; Ohmura and Friese, 1985). Hirota et al., showed that in rat pituitary cells, the redistribution of protein kinase C induced by PMA was associated with the stimulation of LH release (Hirota et al., 1986). Recent studies have suggested that translocation of protein kinase C from the cytosol to the membrane is associated
with activation of the enzyme by PMA and hormonal ligands in several tissues (Kraft et al., 1982; Kraft and Anderson, 1983; Tapley and Murray, 1984). Especially in GH₃ cells, Drust and Martin showed that the release of PRL by phorbol ester correlated with the translocation of protein kinase C (Drust and Martin, 1985). In this study, we demonstrated that a decrease in the cytosolic enzyme activity with an increase in the membrane-associated protein kinase C activity occurred following treatment with PMA and diC8 but not arachidonic acid. Although the primary cell culture of rat pituitary is heterologous cell population, the stimulatory effects of PRL release by PMA, diC8 and arachidonic acid have at least some effect on the lactotrophs. These results indicate that the activation of protein kinase C and its translocation from the cytosol to the membrane by PMA and diC8 are a pre-requisite for PRL release, and also that arachidonic acid and 5-HETE have ad-additive effects with PMA in increasing PRL (Figs. 5, 6). These results indicate that PRL release may involve at least two intermediate steps, a 5-HETE-mediated pathway and a protein kinase C-mediated pathway.

There are several reports indicating that the phorbol ester causes the release of arachidonic acid from membrane phospholipids in several tissues (Wertz and Mueller, 1978; Levine and Hassid, 1977; Mufson et al., 1979; Valone et al., 1983). Arachidonic acid is mainly released in three pathways; first in diglyceride induced by diglyceride lipase, second in phosphatidic acid induced by phosphatidic acid specific phospholipase A₂, and third in phospholipid induced by phospholipase A₂. Chang et al., found that the effect of arachidonic acid plus PMA on LH release from rat pituitary cells was equivalent to that of GnRH (Chang et al., 1986). Naor et al., showed that 100 ng/ml phorbol ester did not affect rapid hydrolysis of PI (Naor et al., 1986). Moreover, Canonico et al. found that the effect of PMA was not antagonized by NDGA or BW755C, which inhibited the stimulation of PRL release by blocking the lipoxygenase activity, suggesting that most of the PMA was probably related to protein kinase C (Canonico et al., 1985). Experiments are in progress to determine which is the main metabolic pathway for arachidonic acid release during the PRL release. In this study arachidonic acid and 5-HETE were tested at concentrations of 10⁻⁶—5 x 10⁻⁵ M, which were in the effective ranges previously reported (Naor et al., 1985). These concentrations are difficult to compare with physiological concentrations in the pituitary, so the physiological roles of arachidonic acid at the pituitary level are still unknown. The additive effects of PMA and arachidonic acid on PRL release suggest that the amount of arachidonic acid release induced by PMA is not enough for maximum PRL release. Also, from the present study we suggest that the signals for stimulation of PRL release by arachidonic acid and 5-HETE are different from the signal through protein kinase C. Moreover, the release of other pituitary hormones such as LH, TSH, GH, may work through the same mechanism.

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References


lipoygenase metabolites or arachidonic acid on calcium homeostasis in neutrophils. J. Biol. Chem. 257, 8608–8611.


