Concerted action of cytosolic Ca^{2+} and protein kinase C in receptor-mediated phospholipase D activation in Chinese hamster ovary cells expressing the cholecystokinin-A receptor

Remko R. BOSCH*, Rolf L. L. SMEETS*, Frank SLEUTELS*, Anjana M. P. PATEL†, Sjenet E. VAN EMST-DE VRIES*, Jan Joep H. H. M. DE PONT* and Peter H. G. M. WILLEMS*¹

* Department of Biochemistry, University of Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands, and † Division of Biosciences, University of Hertfordshire, Hatfield, Hertfordshire, U.K.

Receptor-mediated activation of phosphatidylcholine phosphatidohydrolase or phospholipase D (PLD) was studied in Chinese hamster ovary (CHO) cells expressing the cholecystokinin-A (CCK-A) receptor. Cells were labelled with [³H]myristic acid for 24 h and PLD-catalysed [³H]phosphatidylethanol formation was measured in the presence of 1% (v/v) ethanol. Cholecystokinin-(26–33)-peptide amide (CCK₈) increased PLD activity both time- and dose-dependently. Maximal activation of protein kinase C (PKC) with 1 μM PMA or sustained elevation of the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) with 1 μM thapsigargin increased PLD activity to 50% and 70% of the maximal value obtained with CCK₈ respectively. The stimulatory effects of CCK₈, PMA and thapsigargin were abolished in cells in which PKC was downregulated or inhibited by chelerythrine. PMA/ Ca^{2+} -stimulated PLD activity was absent in a homogenate

of PKC-downregulated cells but could be restored upon addition of purified rat brain PKC. CCK₈-induced PLD activation was inhibited by 90% in the absence of external Ca^{2+} , demonstrating that receptor-mediated activation of PKC in itself does not significantly add to PLD activation but requires a sustained increase in $[\text{Ca}^{2+}]_i$. Taken together, the results presented demonstrate that, in CHO-CCK-A cells, receptor-mediated PLD activation is completely dependent on PKC, but that the extent to which PLD becomes activated depends largely, if not entirely, on the magnitude and duration of the agonist-induced increase in $[\text{Ca}^{2+}]_i$.

Key words: chelerythrine, phorbol ester, phospholipase D, staurosporine, thapsigargin.

INTRODUCTION

Many hormones, neurotransmitters and growth factors use lipid-derived signalling molecules to exert their biological effects. A well-elucidated pathway involves the 1-phosphatidylinositol phosphodiesterase [EC 3.1.4.10; phospholipase C (PLC)]-catalysed hydrolysis of inositol phospholipids to yield $\text{Ins}(1,4,5)\text{P}_3$, releasing calcium from the endoplasmic reticulum [1], and 1,2-diacylglycerol (DAG) which activates protein kinase C (EC 2.7.1.37; PKC) [2]. This pathway is activated both via receptors that couple to a G-protein to activate PLC- β and receptors that regulate intrinsic and extrinsic tyrosine kinases to activate PLC- γ [3–5].

It is widely observed that agonists that stimulate the enzymic breakdown of inositol phospholipids to increase the concentration of cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) and activate PKC also promote the hydrolysis of phosphatidylcholine (PtdCho) to yield choline and phosphatidic acid [5,6]. It has been suggested that phosphatidylcholine phosphatidohydrolase [EC 3.1.4.4; phospholipase D (PLD)] is responsible for this agonist-induced breakdown of PtdCho [3]. Indeed, partially purified mammalian PLD has been shown to be highly selective for this phospholipid [3]. Moreover, agonists which stimulate the hydrolysis of PtdCho also cause the formation of phosphatidylalcohols as a result of the PLD-catalysed transphosphatidylat

reaction [7]. To date, the physiological relevance of agonist-induced PtdCho hydrolysis is unclear. However, it has been proposed that phosphatidic acid is rapidly converted into DAG to activate PKC [2].

Although Ca^{2+} and PKC have been identified as important activators of PLD, their relative importance in agonist-induced PLD activation is still poorly defined [6,8,9]. In intact cells, receptor-mediated PLD activation can be mimicked by phorbol esters, which activate PKC, and Ca^{2+} ionophores, which increase $[\text{Ca}^{2+}]_i$ [5,9–11]. It is generally observed that phorbol esters are much more effective than receptor agonists. However, preliminary experiments, using Chinese hamster ovary (CHO) cells stably expressing the cholecystokinin-A receptor (CHO-CCK-A cells), revealed that in this cell type the phorbol ester PMA was less effective than cholecystokinin-(26–33)-peptide amide (CCK₈). This might suggest the presence of an additional pathway in CCK-induced PLD activation. To investigate this possibility, we studied the mechanism of CCK₈-induced PLD activation in CHO-CCK-A in more detail.

The results presented demonstrate that PKC in itself is indispensable for receptor-mediated PLD activation and that the extent to which PLD becomes activated depends largely, if not entirely, on the duration and magnitude of the agonist-induced increase in $[\text{Ca}^{2+}]_i$.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[\text{Ca}^{2+}]_{i,av}$, average cytosolic free Ca^{2+} concentration; CCK-A, cholecystokinin A; CCK₈, cholecystokinin-(26–33)-peptide amide; DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal-calf serum; PAF, platelet-activating factor; PKC, protein kinase C; PLC, phospholipase C (1-phosphatidylinositol phosphodiesterase); PLD, phospholipase D (phosphatidylcholine phosphatidohydrolase); PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanol.

¹ To whom correspondence should be addressed (e-mail P.Willems@bioch.kun.nl).

MATERIALS AND METHODS

Materials

CCK₈, PMA, leupeptin and 2,2,4-trimethylpentane were obtained from Sigma (St. Louis, MO, U.S.A.) and thapsigargin from LC Services (Woburn, MA, U.S.A.). PMSF was purchased from SERVA (Heidelberg, Germany) and [9,10(n)-³H]-myristic acid (53 Ci/mmol) from The Radiochemical Centre (Amersham, U.K.). Staurosporine was obtained from Boehringer (Mannheim, Germany) and chelerythrine from Research Biochemicals International (Köln, Germany). Aprotinin was purchased from Calbiochem (La Jolla, CA, U.S.A.) and dithiothreitol from Research Organics Inc. (Cleveland, OH, U.S.A.). HPTLC silica-gel 60 plates, ethyl acetate and chloroform were obtained from Merck (Darmstadt, Germany), phospholipid standards from Avanti Polar Lipids (Birmingham, AL, U.S.A.), and isotype-specific PKC antibodies and tissue-culture medium with additives from Gibco (Paisley, Scotland, U.K.). Fura-2/acetoxymethyl ester was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). I-block reagent was purchased from Tropix (Bedford, MA, U.S.A.). All other chemicals were of reagent grade.

Development of a stable CHO-CCK-A cell line

The development of a stable CHO-CCK-A cell line has been described in detail previously [12].

PLD measurements in CHO-CCK-A cells

CHO-CCK-A cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum (FCS), in a water-saturated atmosphere containing 5% CO₂ at 37°C. For PLD measurements, the cells were subcultured in 12-well plates (2.2 × 10⁵ cells/well) and labelled with [³H]myristic acid (3 µCi/ml) for 24 h. At the end of the labelling period, the cells were washed and incubated in DMEM without FCS for 3 h. In PKC downregulation experiments, PMA (0.1 µM) was added at the beginning of either the labelling (24 h PMA treatment) or the incubation with FCS-free DMEM (3 h PMA treatment). Subsequently, the cells were washed and incubated in DMEM containing 1% (v/v) ethanol for the indicated period of time. Ca²⁺-free experiments were performed with minimal essential medium to which no Ca²⁺ was added and which contained in addition 0.5 mM EGTA. At the indicated time, the medium was aspirated and the reaction was quenched by the addition of 1 ml of ice-cold methanol. The cells were scraped off and the mixture was transferred into a lipid tube (Sarstedt, Essen, Germany). Lipids were extracted according to the method described by Bligh and Dyer [13]. Briefly, 500 µl of chloroform and 400 µl of water were added and the mixture was mixed vigorously. Phase separation was achieved by addition of 400 µl of chloroform and 400 µl of water followed by centrifugation. After collection of the lower organic phase, the aqueous phase was extracted again and the combined organic phases were blown to dryness with N₂.

Separation of labelled phospholipids

The dried extracts were dissolved in 20 µl of chloroform/methanol (9:1, v/v) and the lipids were separated by TLC using potassium oxalate-treated silica-gel 60 HPTLC plates. The plates were developed with the upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, by vol.). Radioactive spots corresponding to authentic lipid standards were visualized by I₂-staining and scraped off. Radioactivity was measured by liquid-scintillation spectrometry.

PLD measurements in CHO-CCK-A homogenates

PLD activity was measured in cell homogenates according to the method of Ohguchi et al. [14] as described previously [15]. Briefly, CHO-CCK-A cells were grown to confluence in the presence of [³H]myristic acid (2 µCi/ml) for 48 h. Before homogenization, the cells were washed and incubated in DMEM without FCS for 3 h. In PKC downregulation experiments, PMA (0.1 µM) was added either 3 or 24 h before homogenization. At the end of the incubation period, the cells were washed twice with an ice-cold homogenization solution containing 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 5 mM dithiothreitol, 0.5 mM PMSF, 10 µg/ml leupeptin and 25 mM Hepes, adjusted with Tris to pH 7.4 and collected by scraping. The cells were subsequently homogenized at 4°C using a Potter-Elvehjem homogenizer (glass-Teflon) by 10 strokes at 500 rev./min, 5 strokes at 1000 rev./min and 5 strokes at 1200 rev./min. Unbroken cells were removed by centrifugation at 50 g for 5 min. For PLD measurements, aliquots of the homogenate were taken and 0.5 mM Mg-ATP and 1% (v/v) ethanol were added with or without PMA and CaCl₂ at final concentrations of 0.1 µM and 0.5 mM respectively. The homogenate was incubated for 30 min at 37°C. The reaction was quenched with 1 ml of ice-cold methanol and lipids were extracted and separated as described above.

Partially purified PKC from rat brain

Partially purified PKC, to be added to homogenates of CHO-CCK-A cells in which PKC was downregulated, was prepared from rat brain essentially as described previously [16]. Briefly, rat brain tissue was homogenized in a buffer containing 20 mM Tris/HCl (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 0.2 mg/ml soybean trypsin inhibitor, 5 mM dithiothreitol, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin. The homogenate was applied to a protamine-agarose column. Non-specifically bound protein was eluted with a buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 10 mM MgCl₂ and 1 M KCl. Subsequently, PKC was eluted with the same buffer containing 0.7 M KCl and 1 mM ATP. Western blot analysis revealed the presence of PKC-α, -β, -γ, -δ, -ε, -ζ, -η and -θ.

Fluorescence measurements in suspensions of CHO-CCK-A cells

Measurement of the [Ca²⁺]_i in CHO-CCK-A cells has been described in detail previously [17–19]. In brief, cells were loaded with the specific fluorescent calcium indicator, fura-2. Fluorescence measurements were carried out at 37°C and the fluorescence emission ratio was monitored as a measure of [Ca²⁺]_i at 490 nm after excitation at 340 and 380 nm.

PKC-isotype measurements in CHO-CCK-A cells

PKC-isotype measurements were performed as described in detail previously [12,18].

Analysis of the data

The results presented are the means ± S.E.M. of the number of experiments indicated in the text. Paired Student's *t*-tests were used to determine statistical differences (*P* < 0.05).

RESULTS

Time course of CCK₈- and PMA-stimulated [³H]phosphatidylethanol (PtdEth) formation in CHO-CCK-A cells

Both the peptide hormone CCK₈ and the phorbol ester PMA increased the amount of [³H]PtdEth time-dependently when added to cells labelled with [³H]myristic acid and incubated in the presence of 1% (v/v) ethanol (Figure 1). The time course of the stimulatory effect of 10 nM CCK₈ revealed that the rate of [³H]PtdEth formation was highest during the first 3 min of stimulation, after which it decreased to the significantly lower levels reached at 10 min and maintained for at least another 20 min. CCK₈-induced [³H]PtdEth formation was dose-dependent and maximal stimulation was obtained with 10 nM CCK₈ (results not shown).

With PMA the rate of [³H]PtdEth formation was relatively constant during the first 10 min and, depending on the PMA concentration, either remained constant (0.1 μ M), decreased markedly (0.3 μ M), or even fell back to zero (1 μ M), thereafter. This finding shows that the PMA response rapidly desensitized with higher PMA concentrations. Compared with CCK₈, the effect of PMA (1 μ M) was significantly lower at all three measuring points. When added simultaneously, PMA (1 μ M) neither potentiated nor inhibited the effect of 10 nM CCK₈. This demonstrates that the mechanism underlying desensitization of the PMA response did not affect the CCK₈ response.

Comparison of CCK₈-, thapsigargin- and PMA-stimulated [³H]PtdEth formation in CHO-CCK-A cells

In addition to stimulating PLD, 10 nM CCK₈ evoked a rapid and sustained increase in the average cytosolic free Ca²⁺ concentration ([Ca²⁺]_{i,av}) in a suspension of CHO-CCK-A cells

Figure 1 Time-dependence of CCK₈- and PMA-stimulated formation of [³H]PtdEth in CHO-CCK-A cells

CHO-CCK-A cells, labelled with [³H]myristic acid for 24 h, were stimulated with 10 nM CCK₈ (○), 0.1 μ M PMA (◆), 0.3 μ M PMA (▼), 1 μ M PMA (■) or the combination of 10 nM CCK₈ and 1 μ M PMA (●) in the presence of 1% (v/v) ethanol. At the indicated times, the reaction was quenched by the addition of ice-cold extraction medium. The lipids were separated by HPTLC, radioactive spots were scraped off and radioactivity was measured by liquid-scintillation spectroscopy. In each experiment, the amount of [³H]PtdEth present in cells stimulated with 10 nM CCK₈ alone for 30 min is set at 100%, to which all other values are related. The values presented are the mean $\pm$ S.E.M. of 3–6 experiments. At all three measuring points the values obtained with PMA alone were significantly different ($P < 0.05$) from those obtained with CCK₈ alone. TPA is PMA.

Figure 2 Extracellular Ca²⁺-dependence of the CCK₈-induced increase in [Ca²⁺]_{i,av} in a suspension of CHO-CCK-A cells

CHO-CCK-A cells, loaded with fura-2, were resuspended in either normal or Ca²⁺-free HEPES/Tris medium (pH 7.4) and transferred to a cuvette placed in a spectrofluorophotometer. Ca²⁺-free medium contained in addition 0.5 mM EGTA. At the indicated time, the cells were stimulated with either 1 μ M thapsigargin or 10 nM CCK₈ and the fluorescence emission ratio at 490 nm was monitored as a measure of the [Ca²⁺]_{i,av} after excitation at 340 and 380 nm.

(Figure 2). To study the relative importance of this sustained increase in [Ca²⁺]_i in CCK₈-stimulated [³H]PtdEth formation, cells were stimulated with the sesquiterpene lactone thapsigargin. Thapsigargin inhibits the endoplasmic reticulum Ca²⁺-ATPase [20], leading to depletion of intracellular Ca²⁺ stores and thus to an increase in store-regulated Ca²⁺ entry [21]. As a result, [Ca²⁺]_i remains elevated for a prolonged period of time (Figure 2). Figure 3 shows that 1 μ M thapsigargin alone markedly stimulated the formation of [³H]PtdEth. After correction for basal [³H]PtdEth formation, the thapsigargin-induced increase in [³H]PtdEth was calculated to be 70% of the value obtained with 10 nM CCK₈. The effect of thapsigargin was not significantly different from that of PMA, which amounted to 50% of the value obtained with 10 nM CCK₈. The stimulatory effects of 1 μ M thapsigargin and 1 μ M PMA were more than additive and the amount of [³H]PtdEth formed as a result of the combined action of both drugs was 1.4-fold higher than that obtained with 10 nM CCK₈. Interestingly, PMA, when added at a 1000-fold lower concentration of 1 nM, did not increase PLD activity by itself, but significantly potentiated the stimulatory effect of 1 μ M thapsigargin to 100% (S.E.M. = 10, n = 3) of the value obtained with 10 nM CCK₈.

Effect of protein kinase inhibitors on CCK₈-, thapsigargin- and PMA-stimulated [³H]PtdEth formation in CHO-CCK-A cells

Both staurosporine (0.1 μ M), a general inhibitor of protein kinase activity [22], and chelerythrine (50 μ M), a specific inhibitor of PKC [23], completely inhibited the stimulatory effect of PMA (Figure 3). However, when added in combination with either CCK₈, thapsigargin or the combination of PMA and thapsigargin, staurosporine invariably reduced the increase in [³H]PtdEth above the basal level to 30% of the value obtained with CCK₈ alone, whereas chelerythrine virtually completely blocked [³H]PtdEth formation under all stimulatory conditions. The inhibitors did not significantly affect basal [³H]PtdEth formation.

Figure 3 Inhibitory effect of staurosporine and chelerythrine on CCK₈-, PMA- and thapsigargin-induced [³H]PtdEth formation in CHO-CCK-A cells

CHO-CCK-A cells, labelled with [³H]myristic acid for 24 h, were incubated in the absence (white bars) or presence of either 0.1 μM staurosporine (black bars) or 50 μM chelerythrine (hatched bars) for 5 min and subsequently stimulated with 10 nM CCK₈, 1 μM PMA, 1 μM thapsigargin (THAPS) or the combination of 1 μM PMA and 1 μM thapsigargin in the presence of 1% (v/v) ethanol for 10 min. The reaction was quenched at the indicated time and the lipids were separated by HPTLC. Radioactive spots were scraped off and radioactivity was measured by liquid-scintillation spectroscopy. In each experiment, the amount of [³H]PtdEth present in cells stimulated with 10 nM CCK₈ in the absence of drug is set at 100%, to which all other values are related. The values presented are the means ± S.E.M. of 3–5 experiments. *Significantly different ($P < 0.05$) from corresponding unstimulated cells. #Significantly different ($P < 0.05$) from corresponding staurosporine-treated cells. TPA is PMA.

Extracellular Ca²⁺-dependence of CCK₈- and PMA-stimulated [³H]PtdEth formation in CHO-CCK-A cells

CCK₈ (10 nM) transiently increased [Ca²⁺]_{i,av} in a suspension of CHO-CCK-A cells incubated in the absence of extracellular Ca²⁺ (Figure 2). Under these conditions the increase in [³H]PtdEth in response to 10 nM CCK₈ was dramatically reduced by 87.8% (S.E.M. = 2.5, $n = 4$). In contrast, the stimulatory effect of 1 μM PMA was even enhanced in the absence of external Ca²⁺ by 63.4% (S.E.M. = 9.8, $n = 4$).

Effect of PKC downregulation on CCK₈-, PMA- and thapsigargin-induced [³H]PtdEth formation in CHO-CCK-A cells

Immunoblotting revealed that CHO-CCK-A cells contained detectable amounts of PKC-α, PKC-ε and PKC-ζ. These three PKC isotypes were differentially downregulated during prolonged PMA (0.1 μM) treatment. The amount of PKC-α was decreased by 94% after 3 h of incubation in the presence of PMA, whereas PKC-ε was maximally decreased after 24 h of PMA treatment (Table 1). In contrast, the amount of PKC-ζ remained unchanged. The preferential decrease in PKC-α was accompanied by a rapid decrease in CCK₈-stimulated [³H]PtdEth formation. The response to 10 nM CCK₈ was abolished 3 h after the onset of PMA treatment. Similarly, the stimulatory effect of thapsigargin was completely inhibited at 3 h of PMA treatment.

Effect of partially purified PKC on PMA/Ca²⁺-stimulated PLD activity in homogenates of PKC-downregulated CHO-CCK-A cells

Preliminary experiments to determine the optimal concentrations of PMA and CaCl₂ revealed that with CaCl₂ alone a maximal increase above basal (65% of the value obtained with 0.1 μM PMA and 0.5 mM CaCl₂) was obtained at a concentration of

Table 1 Effect of long-term PMA treatment on CCK₈-, PMA- and thapsigargin-induced [³H]PtdEth formation and PKC-α, -ε and -ζ levels in CHO-CCK-A cells

CHO-CCK-A cells, labelled with [³H]myristic acid for 24 h, were incubated in the absence (0 h PMA) or presence of 0.1 μM PMA for either 3 h or 24 h. Subsequently, cells were stimulated with 10 nM CCK₈, 0.1 μM PMA or 1 μM thapsigargin in the presence of 1% (v/v) ethanol for 10 min. The reaction was quenched at the indicated time and the lipids were separated by HPTLC. Radioactive spots were scraped off and radioactivity was measured by liquid-scintillation spectroscopy. In each experiment, the amount of [³H]PtdEth present in untreated cells stimulated with 10 nM CCK₈ is set at 100%, to which all other values are related. Except for thapsigargin and 24 h PMA, the values presented are the means ± S.E.M. of three experiments. For PKC-isotype measurements, total cell lysates were subjected to SDS/PAGE followed by Western blotting. Membranes were incubated with PKC-isotype-specific antibodies in the presence (aspecific staining) and absence (total staining) of the corresponding isotype-specific peptides. PKC-specific bands were quantified densitometrically. In each experiment, the mean absorbance of the untreated control is set at 100%, to which all other values are related. The results presented are the means ± S.E.M. of three experiments. *Significantly different ($P < 0.05$) from corresponding unstimulated cells. #Significantly different ($P < 0.05$) from corresponding untreated (0 h PMA) cells. n.d., not determined.

	0 h PMA	3 h PMA	24 h PMA
Relative PLD activity			
Unstimulated	11.6 ± 1.2	—	—
PMA	31.0 ± 3.1*	13.0 ± 2.1#	9.1
CCK ₈	100*	15.3 ± 2.3#	10.7
PMA + CCK ₈	114 ± 23*	—	—
Thapsigargin	83	15.3	n.d.
Relative PKC levels			
PKC-α	100	6.0 ± 3.6#	3.3 ± 3.3#
PKC-ε	100	68.7 ± 18.5	18.8 ± 4.0#
PKC-ζ	100	90.0 ± 13.8	101.2 ± 4.8

0.5 mM. In the presence of 0.5 mM CaCl₂, PMA further increased PLD activity to the maximum reached at 0.1 μM. The increase above basal levels with 0.1 μM PMA alone amounted to only 13% of the value obtained with 0.1 μM PMA and 0.5 mM CaCl₂. Importantly, this activation profile of PLD is identical with that of PKC.

Figure 4 shows that addition of 0.1 μM PMA and 0.5 mM CaCl₂ resulted in a 2.5-fold increase in PLD activity in a homogenate of CHO-CCK-A cells. PMA/Ca²⁺-stimulated PLD activity depended largely, but not entirely, on ATP, since without its addition only a 1.4-fold (S.E.M. = 0.1, $n = 4$) increase in PLD activity was observed. PMA/Ca²⁺-stimulated PLD activity was dramatically decreased in homogenates of cells pretreated with PMA for 3 h and abolished after 24 h of phorbol ester treatment (Figure 4). Addition of partially purified PKC from rat brain did not affect PMA/Ca²⁺-stimulated PLD activity in untreated cells, while virtually completely restoring this activity in homogenates from PMA-treated cells. Addition of PKC had no significant effect on PLD activity measured in the absence of PMA and CaCl₂.

DISCUSSION

The present study demonstrates that CCK₈ increases PLD activity in CHO-CCK-A cells solely through PKC and an increase in [Ca²⁺]_i, and that no other pathway is involved. This conclusion is based on the following observations: (i) PKC, most probably PKC-α, is indispensable for receptor-mediated PLD activation; (ii) receptor-mediated PKC activation in itself hardly increases PLD activity; and (iii) the extent to which PLD becomes activated during receptor-mediated PLD activation depends largely, if not completely, on the duration and magnitude of the agonist-induced increase in [Ca²⁺]_i.

Figure 4 Effect of long-term PMA treatment on PMA- and Ca^{2+} -induced formation of $[^3\text{H}]\text{PtdEth}$ in CHO-CCK-A homogenates

CHO-CCK-A cells, labelled with $[^3\text{H}]$ myristic acid for 24 h, were incubated in the absence (0 h PMA) or presence of $0.1 \mu\text{M}$ PMA for either 3 h or 24 h. Subsequently, cells were homogenized and homogenates were stimulated with $0.1 \mu\text{M}$ PMA and 0.5 mM CaCl_2 in the presence of 1% (v/v) ethanol and in the absence or presence of purified rat brain PKC for 30 min. The reaction was quenched at the indicated time and the lipids were separated by HPTLC. Radioactive spots were scraped off and radioactivity was measured by liquid-scintillation spectroscopy. In each experiment, the amount of $[^3\text{H}]\text{PtdEth}$ formed in homogenates from untreated cells following incubation in the presence of PMA and Ca^{2+} is set at 100%, to which all other values are related. The values presented are the means $\pm$ S.E.M. of four experiments. *Significantly different ($P < 0.05$) from unstimulated conditions with homogenate of untreated cells. TPA is PMA.

(i) Evidence for the absolute requirement of CCK_8 -induced PLD activation for PKC comes from the following observations. First, neither CCK_8 nor PMA increased PLD activity in PKC downregulated cells, notwithstanding the fact that CCK_8 , as has been demonstrated previously [12], causes a sustained increase in $[\text{Ca}^{2+}]_i$ in PMA-treated cells. Consistent with the latter finding, thapsigargin did not increase PLD activity in PKC downregulated cells. Secondly, the PKC inhibitor chelerythrine completely blocked the stimulatory effects of CCK_8 , PMA, thapsigargin and the combination of the latter two drugs. Thirdly, PMA/ Ca^{2+} -stimulated PLD activity was completely inhibited in a homogenate of PKC-downregulated cells but could be restored after addition of partially purified PKC from rat brain.

An absolute requirement of agonist-, phorbol ester- and Ca^{2+} -induced PLD activation for PKC has been reported in a wide variety of cell types [24–27], including CHO cells expressing the platelet-activating factor (PAF) receptor (CHO-PAF cells) [28]. Noticeably, these studies did not take into account the possibility that prolonged treatment with phorbol ester might have led to downregulation of factors other than PKC that may play a role in receptor-mediated PLD activation. However, given the finding that the effect of CCK_8 on PLD activity depended largely on its effect on $[\text{Ca}^{2+}]_i$, the observation that PMA/ Ca^{2+} -stimulated PLD activity in a homogenate of PKC-downregulated cells was completely restored after addition of partially purified PKC does not support this possibility.

Evidence has been provided that AlF_4^- -insensitive monomeric G-proteins, such as ARF and Rho, can directly activate PLD [29]. Thus far, however, the mechanism by which agonists activate these small G-proteins is unknown. In a recent study it was demonstrated that AlF_4^- stimulation of PLD activity in Saos-2 cells through heterotrimeric G-proteins was accompanied by redistribution of ARF and RhoA to the membrane [30]. Since the effects of AlF_4^- and phorbol ester were more than additive, and preferential downregulation of $\text{PKC-}\alpha$ by chronic phorbol

ester treatment abolished the effect of the phorbol ester, but not that of AlF_4^- , it was proposed that these small G-proteins act synergistically with PKC in receptor-mediated PLD activation. Of note is the similar additive effect between agonist and phorbol ester that was observed in CHO cells expressing the V1a vasopressin receptor (CHO-V1a cells) and regarded as indicative of the existence of different mechanisms of PLD activation [31]. However, the present finding that preferential downregulation of $\text{PKC-}\alpha$ completely inhibited the action of CCK_8 supports the idea that no factors other than PKC are involved in agonist-induced PLD activation in CHO-CCK-A cells.

Immunoblot analysis revealed that the loss of PMA- and CCK_8 -induced PLD activation, as observed during chronic PMA treatment, correlated best with the disappearance of $\text{PKC-}\alpha$. In contrast with this study, preferential downregulation of $\text{PKC-}\alpha$ in CHO-PAF cells by 6 h exposure to $0.1 \mu\text{M}$ phorbol ester has previously been found not to completely inhibit agonist- and phorbol ester-induced PLD activation [32]. Similarly, in CHO-V1a cells, 3 h exposure to $0.1 \mu\text{M}$ phorbol ester did not even reduce agonist-induced PLD activation by half [31], whereas the effect of the phorbol ester was virtually completely inhibited [31,33]. The possibility that the V1a receptor activates an additional PKC-independent pathway leading to PLD activation is unlikely, since agonist-induced PLD activation was completely inhibited by calphostin-C [31].

Intriguingly, despite the fact that CCK_8 , thapsigargin and the combination of PMA and thapsigargin increased PLD activity to a different extent, staurosporine invariably reduced stimulated PLD activity to 30% of the value obtained with CCK_8 alone. Given the absolute requirement for PKC and the fact that staurosporine acts as a competitive inhibitor at the ATP-binding site [22], this suggests that the greater part of the agonist-induced increase in PLD activity occurs in a phosphorylation-dependent manner. The smaller staurosporine-resistant part may then reflect the ATP-independent PKC-mediated pathway originally postulated by Conricode et al. [34]. In agreement with this idea, PMA/ Ca^{2+} -stimulated PLD activity in a homogenate of CHO-CCK-A cells was shown to depend largely, but not entirely, on the presence of ATP in the assay medium. Also in agreement with this idea, chelerythrine, which acts in a competitive fashion with respect to the phosphate acceptor [23], completely inhibited the CCK_8 -induced increase in PLD activity. Staurosporine abolished PMA-induced PLD activation, suggesting that the phorbol ester acts entirely via the phosphorylation-dependent pathway. This then suggests that the ATP-independent pathway is activated by the sustained increase in $[\text{Ca}^{2+}]_i$ observed with CCK_8 and thapsigargin.

(ii) The observation that in the absence of external Ca^{2+} the effect of CCK_8 was inhibited by 90% demonstrates that receptor-mediated PKC activation in itself only marginally contributes to agonist-induced PLD activation. The absence of external Ca^{2+} prevented $[\text{Ca}^{2+}]_i$ from being increased for prolonged periods of time, demonstrating that a sustained increase in $[\text{Ca}^{2+}]_i$ is required for full activation of PLD. In contrast with the present study, agonist and phorbol ester displayed the same maximal effect in CHO-V1a [31] and CHO-PAF [28] cells. In view of the above discussion, however, this discrepancy might be explained by the higher magnitude of the sustained increase in $[\text{Ca}^{2+}]_i$ in CHO-CCK-A cells as compared with CHO-V1a cells.

(iii) The finding that thapsigargin increased PLD activity to 70% of the value obtained with 10 nM CCK_8 suggests that only 30% of receptor-induced PLD activation depends on DAG-mediated PKC activation. This is in agreement with the observation that low-level PKC activation by 1 nM PMA, which did not affect PLD activity by itself, potentiated the stimulatory

effect of thapsigargin to a level similar to that obtained with 10 nM CCK₈. The thapsigargin response was further increased to 138% of the value obtained with 10 nM CCK₈ by a PMA concentration (1 μ M) that was maximally effective in stimulating PLD activity by itself. Surprisingly, the response to the latter PMA concentration was also increased under conditions when [Ca²⁺]_i was most likely to be decreased as a result of the omission of Ca²⁺ from the medium. At present, the reason for this apparent discrepancy is unclear.

Taken together, the present observations are consistent with the idea that agonist-induced PLD activation in CHO-CCK-A cells occurs in a PKC-dependent manner, most probably involving both a phosphorylating and a non-phosphorylating mechanism. Despite its indispensability, however, the contribution of PKC activation alone in receptor-mediated PLD activation is low compared with that of the increase in [Ca²⁺]_i.

The work of R.R.B. was supported by a grant from the Netherlands Organization for Scientific Research. The research of P.H.G.M.W. has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

REFERENCES

- Berridge, M. J. (1993) *Nature* (London) **361**, 315–325
- Nishizuka, Y. (1992) *Science* **258**, 607–614
- Exton, J. H. (1994) *Annu. Rev. Physiol.* **56**, 349–369
- Noh, D.-Y., Shin, S. H. and Rhee, S. G. (1995) *Biochim. Biophys. Acta* **1242**, 99–114
- Natarajan, V., Scribner, W. M. and Vepa, S. (1996) *Chem. Phys. Lipids* **80**, 103–116
- Exton, J. H. (1994) *Biochim. Biophys. Acta* **1212**, 26–42
- Dawson, R. M. C. (1967) *Biochem. J.* **102**, 205–210
- Exton, J. H. (1997) *Eur. J. Biochem.* **243**, 10–20
- Cockcroft, S. (1997) *Prog. Lipid Res.* **35**, 345–370
- Kiss, Z. (1996) *Chem. Phys. Lipids* **80**, 81–102
- Morris, A. J., Engebrecht, J. and Frohmann, M. A. (1996) *Trends Pharmacol. Sci.* **17**, 182–185
- Smeets, R. L. L., Garner, K. M., Hendriks, M., Van Emst-De Vries, S. E., Peacock, M. D., Hendriks, W., De Pont, J. J. H. H. M. and Willems, P. H. G. M. (1996) *Cell Calcium* **20**, 1–9
- Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Oguchi, K., Banno, Y., Nakashima, S. and Nozawa, Y. (1996) *J. Biol. Chem.* **271**, 4366–4372
- Bosch, R. R., Patel, A. M. P., Van Emst-De Vries, S. E., Smeets, R. L. L., De Pont, J. J. H. H. M. and Willems, P. H. G. M. (1998) *Eur. J. Pharmacol.* **346**, 345–351
- Ederveen, A. G. H., Van Emst-De Vries, S. E., Burgers, L. H. C. and De Pont, J. J. H. H. M. (1992) *Pancreas* **7**, 34–44
- Smeets, R. L. L., Ijzerman, A. P., Hermesen, H. P. H., Ophorst, O. J. A. E., Van Emst-De Vries, S. E., De Pont, J. J. H. H. M. and Willems, P. H. G. M. (1997) *Eur. J. Pharmacol.* **325**, 93–99
- Smeets, R. L. L., Fouraux, M. A., Van Emst-De Vries, S. E., De Pont, J. J. H. H. M. and Willems, P. H. G. M. (1998) *Br. J. Pharmacol.* **123**, 1189–1197
- Smeets, R. L. L., Fouraux, M. A., Pouwels, W., Van Emst-De Vries, S. E., Ronken, E., De Pont, J. J. H. H. M. and Willems, P. H. G. M. (1998) *Br. J. Pharmacol.* **124**, 935–945
- Lytton, J., Westlin, M. and Hanley, M. R. (1991) *J. Biol. Chem.* **266**, 17067–17071
- Putney, Jr., J. W. and Bird, St. J. G. (1993) *Endocr. Rev.* **14**, 610–631
- Rüegg, U. T. and Burgess, G. M. (1989) *Trends Pharmacol. Sci.* **10**, 218–220
- Herbert, J. M. M., Augereau, J. M. and Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* **172**, 993–999
- Gustavsson, L., Moehren, G., Torres-Marquez, M. E., Benistant, C., Rubin, R. and Hoek, J. B. (1994) *J. Biol. Chem.* **269**, 849–859
- Zheng, L., Stojilkovic, S. S., Hunyady, L., Krstanovic, L. Z. and Catt, K. J. (1994) *Endocrinology* (Baltimore) **134**, 1446–1454
- Balboa, M. A., Firestein, B. L., Godson, C., Bell, K. S. and Insel, P. A. (1994) *J. Biol. Chem.* **269**, 10511–10526
- Pfeilschifter, J. and Huwiler, A. (1993) *FEBS Lett.* **331**, 267–271
- Liu, B., Nakashima, S., Kanoh, H., Takano, T., Shimizu, T. and Nozawa, Y. (1994) *J. Biochem. (Tokyo)* **116**, 882–891
- Hammond, S. M., Altshuler, Y. M., Sung, T.-C., Rudge, S. A., Rose, K., Engebrecht, J., Morris, A. J. and Frohman, M. A. (1995) *J. Biol. Chem.* **270**, 29640–29643
- Bourgoin, S. G., Harbour, D. and Poubelle, P. E. (1996) *J. Bone Miner. Res.* **11**, 1655–1665
- Garces, Y., Briley, E. M. and Felder, C. C. (1995) *Neuropeptides* **28**, 277–285
- Liu, B., Nakashima, S., Takano, T., Shimizu, T. and Nozawa, Y. (1995) *Biochem. Biophys. Res. Commun.* **214**, 418–423
- Dunlop, M. and Clark, S. (1993) *Biochim. Biophys. Acta* **1220**, 43–48
- Conricode, K. M., Brewer, K. A. and Exton, J. H. (1992) *J. Biol. Chem.* **267**, 7199–7202