The regulation of phospholipase D activity and its role in *sn*-1,2-diradylglycerol formation in bombesin- and phorbol 12-myristate 13-acetate-stimulated Swiss 3T3 cells

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Addition of the phorbol ester phorbol 12-myristate 13-acetate (PMA) to quiescent Swiss 3T3 cells resulted in a sustained increase in *sn*-1,2-diradylglycerol (DG) mass and [^3H]DG in [^3H]palmitate-labelled cells where phosphatidylcholine was the major labelled phospholipid. This occurred in the absence of inositol phosphate accumulation. In [^3H]palmitate-labelled cells both bombesin and PMA stimulated the formation of phosphatidylbutanol ([^3H]PtdBut) in the presence of 0.3 % (v/v) butan-1-ol. The kinetics of [^3H]PtdBut formation were consistent with phospholipase D (PLD) activation preceding sustained DG formation. The inclusion of butan-1-ol inhibited 70 % of PMA-stimulated DG formation but only 30 % of the bombesin response. The ability of bombesin and PMA to stimulate the accumulation of [^3H]PtdBut was completely abolished in Swiss 3T3 cells which had been pre-treated with 400 nM-PMA for 48 h to down-regulate protein kinase C activity. PMA-stimulated [^3H]PtdBut formation was inhibited by 90 % by the protein kinase C inhibitor Ro-31-8220 (10 μM), but bombesin-stimulated PtdBut accumulation was inhibited by at most 50 % by the same concentration of inhibitor. Cyclic AMP-elevating agents, i.e. forskolin, dibutyryl cyclic AMP and isobutylmethylxanthine, did not inhibit bombesin stimulation of PLD activity. Bombesin-stimulated PLD activity was inhibited by 50 % by buffering of the extracellular Ca^{2+} concentration to 150 nM, but combination of this treatment with Ro-31-8220 addition was less than additive. Ionophore A23187 alone was able to stimulate PLD activity, but this response was inhibited 50 % by Ro-31-8220. Thapsigargin was unable to stimulate PLD activity and had no modulatory effect upon bombesin-stimulated PLD activity at any agonist concentration. The results are discussed in terms of the role of PLD in DG generation and the regulation of PLD activity both by bombesin and by PMA.

INTRODUCTION

Bombesin exemplifies a family of mitogenic growth factors including α -thrombin, vasopressin and prostaglandin $\text{F}_{2\alpha}$ which bind to their cognate receptors, and, through the mediation of a GTP-binding protein, G_p , stimulate the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by phosphoinositidase C (reviewed by Berridge, 1987; Milligan & Wakelam, 1989). The second messenger products of this reaction are both implicated in the control of cellular proliferation: $\text{Ins}(1,4,5)\text{P}_3$ mobilizes Ca^{2+} from an intracellular store and *sn*-1,2-diradylglycerol (DG) activates protein kinase C (PKC) (reviewed by Berridge, 1987). Whereas a role for an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in cell activation is well known, $\text{Ins}(1,4,5)\text{P}_3$ -mediated increases in $[\text{Ca}^{2+}]_i$ are short-lived. In contrast, many of the early, pre-replicative, events such as increases in pH_i (Bierman *et al.*, 1990), and *c-fos* and *c-myc* expression (Rozengurt & Sinnett-Smith, 1987) occur after $[\text{Ca}^{2+}]_i$ had declined and are sustained or require the continued presence of the growth factor. Furthermore, in Balb/c 3T3 cells, micro-injection of DG, but not $\text{Ins}(1,4,5)\text{P}_3$, is mitogenic (Suzuki-Sekimori *et al.*, 1989), whereas in other cell lines growth factors can activate PKC and exert their mitogenic effect without increasing $\text{Ins}(1,4,5)\text{P}_3$ levels (e.g. Imamura *et al.*, 1990; Wright *et al.*, 1990). These observations suggest that the DG/PKC arm of the signalling pathway can be dissociated

from inositol lipid hydrolysis and may represent a more persistent mitogenic signal.

In addressing these anomalies we sought to define the kinetics of $\text{Ins}(1,4,5)\text{P}_3$ and DG formation in bombesin-stimulated Swiss 3T3 fibroblasts, and have shown that bombesin stimulates a biphasic increase in DG mass in Swiss 3T3 cells (Cook *et al.*, 1990); the second phase is sustained for up to 4 h (Takuwa *et al.*, 1987a, 1989) and is divorced from increases in $\text{Ins}(1,4,5)\text{P}_3$ mass, which declines to control values within 30 s (Cook *et al.*, 1990). Similar results have also been reported in α -thrombin-stimulated IIC9 fibroblasts (Wright *et al.*, 1988). Recent reports have suggested a role for phosphatidylcholine (PtdCho) hydrolysis as an alternative source of DG in a variety of stimulus-response systems (reviewed by Billah & Anthes, 1990). We have described bombesin- and phorbol 12-myristate 13-acetate (PMA)-stimulated PtdCho hydrolysis by an apparent PKC-dependent phospholipase D (PLD) activity in Swiss 3T3 cells based on the rapid release of [^3H]choline ([^3H]Cho) from [^3H]Cho-labelled Swiss 3T3 cells prior to any increases in [^3H]phosphocholine ([^3H]ChoP). However, measuring the release of [^3H]Cho or [^3H]ChoP in isotopically labelled cells is not an unequivocal assay for either PLD or phospholipase C (PLC) respectively; increases in Cho may be due to base-exchange activity with no net formation of phosphatidic acid, and increases in ChoP may result from increased choline kinase activity.

Abbreviations used: DG, *sn*-1,2-diradylglycerol; DMEM, Dulbecco's modified Eagle's medium; DMBH, DMEM containing 1 % BSA and 20 mM-Hepes; G_p , the guanine-nucleotide-binding protein coupling receptors to phospholipase C; HBG, Hanks buffered saline, pH 7.4, containing 10 mM-glucose and 1 % BSA; IBMX, isobutylmethylxanthine; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PPH, phosphatidate phosphohydrolase; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; PtdCho, phosphatidylcholine; ChoP, phosphocholine; PtdBut, phosphatidylbutanol; EC_{50} , concn. giving 50 % of maximal effect; IC_{50} , concn. giving 50 % of maximal inhibition.

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In the present paper we show that the phorbol ester PMA increases the cellular DG content of Swiss 3T3 cells in a sustained manner which appears to mimic the second phase of bombesin-stimulated increases in DG. Furthermore, we confirm that both bombesin and PMA do stimulate PLD activity, as measured by the formation of phosphatidylbutanol (PtdBut), a marker for PLD activity in whole cells (Pai *et al.*, 1988a,b). The kinetics of this PLD activation provide strong evidence for it being the source of sustained increases in DG, but the role of a PLD/phosphatidate phosphohydrolase (PPH) pathway in DG formation differs markedly between the two agonists. In addition, we assess the roles of PKC, protein kinase A (PKA) and Ca^{2+} in the regulation of PLD.

MATERIALS AND METHODS

Cell culture

Swiss 3T3 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % newborn calf serum, 27 mg of glutamine/ml and penicillin/streptomycin (250 units/ml and 250 $\mu\text{g}/\text{ml}$ respectively) at 37 °C in a humidified atmosphere of air/ CO_2 (19:1). All experiments were performed upon serum-starved cells which had been grown to confluency for 36–48 h in 2 % calf serum. For labelling studies [9,10(n)- ^3H]palmitic acid, 4 μCi in 2 ml was included in the 2 %-serum-containing medium for experiments on 6-well plates, and 2 μCi in 0.5 ml for 24-well plates. In all cases, the cells were confluent, quiescent and labelled to equilibrium when used.

Assay of DG mass

Confluent monolayers of Swiss 3T3 cells grown on 6-well plates were washed in serum-free DMEM containing 20 mM-Hepes (pH 7.4) and 1 % (w/v) BSA (DMBH) for 1 h before stimulation with bombesin or PMA for the times and at the concentrations indicated. Incubations were terminated by removal of the medium prior to addition of 0.7 ml of ice-cold methanol, and the harvested cell debris was extracted into 0.7 ml of chloroform on ice for 30 min. Phases were resolved by addition of 0.585 ml of water. An aliquot of the chloroform phase was dried down *in vacuo* in glass vials and solubilized in a Triton X-100/phosphatidylserine (PtdSer) micelle preparation [final concentration 3 mol % phosphatidylserine in 0.3 % (w/v) Triton X-100], and DG mass was determined by the radioenzymic assay of Preiss *et al.* (1986) with the modifications outlined previously (Cook *et al.*, 1990). It should be noted that the values obtained by this method are known to underestimate DG mass (A. Paterson, R. Plevin & M. Wakelam, unpublished work).

Measurement of total inositol phosphates

Quiescent Swiss 3T3 cells labelled with [^3H]inositol were washed in Hanks buffered saline solution, pH 7.4, containing 10 mM-glucose and 1 % (w/v) BSA (HBG) for 5 min at 37 °C. They were then washed in HBG containing 10 mM-LiCl (HBG/Li) for a further 15 min at 37 °C prior to treatment with the appropriate agent. After this pre-incubation period, cells were treated with the given concentration of agonist for the times indicated in HBG/Li at 37 °C. Incubations were terminated and total inositol phosphate formation was assayed as described (Cook & Wakelam, 1991).

Assay of PLD transferase activity in whole cells

Swiss 3T3 cells grown in 6-well plates until 80 % confluent were cultured for a further 48 h in 2 ml of DMEM + 1 % (v/v) newborn calf serum containing a total of 4 μCi of [^3H]palmitic acid/well. Cells were washed in 1 ml of serum-free DMBH for

30 min at 37 °C prior to incubation for a further 5 min in 1 ml of DMBH containing 30 mM-butan-1-ol (0.3 %, v/v). Incubations were started by replacing the medium with 1 ml of DMBH/0.3 % butan-1-ol and the test reagent at the concentrations and for the times indicated at 37 °C. Incubations were terminated by removal of the medium and addition of 0.7 ml of ice-cold methanol. The cells were harvested and extracted in glass vials for 15 min at room temperature with an equal volume of chloroform in a single phase. Phases were resolved by addition of 0.585 ml of water and centrifugation at 2500 *g* for 5 min. The lower chloroform phase was dried down in glass vials *in vacuo* and redissolved in 75 μl of chloroform/methanol (19:1, v/v) with vortex-mixing. The entire sample was applied to a Whatman LK5DF t.l.c. plate which was then developed in the organic phase of 2,2,4-trimethylpentane/ethyl acetate/acetic acid/water (5:11:2:10, by vol.) using an unlined chromatography tank. [^3H]PtdBut, identified by its co-migration with an authentic [^{14}C]PtdBut standard, was scraped off the plates and its radioactivity determined by scintillation counting. Some experiments were performed upon 24-well plates labelled as described above using 20 mM-Hepes-buffered HBG and gave essentially the same results.

Analysis of [^3H]DG levels

[^3H]Palmitate-labelled Swiss 3T3 cells were stimulated with 100 nM-PMA as described for the PLD transferase assay, except that butan-1-ol was excluded from the DMBH. Lipids were extracted in chloroform/methanol as described above for [^3H]PtdBut, and [^3H]DG was isolated by t.l.c. on LK5DF plates with a solvent of hexane/diethyl ether/acetic acid (60:40:1, by vol). [^3H]DG, resolved from 1,3-diradylglycerol and identified by I_2 staining of a standard run in parallel, was scraped off the plates and its radioactivity determined by scintillation counting.

RESULTS

The addition of 100 nM-PMA to confluent quiescent Swiss 3T3 cells resulted in an increase in DG mass which was apparent at 1 min and significant after a 2 min stimulation ($P < 0.01$). DG mass increased until between 5 and 15 min, when the maximum response was attained, but was then sustained above control values (β -phorbol in 0.1 % dimethyl sulphoxide) for 30 min (Fig. 1a) and up to 1 h (results not shown), at which time the level of DG was still comparable with that at 15 min. Incubation with the vehicle control or β -phorbol did not significantly alter DG levels in any experiments performed. The increase in DG mass was dose-dependent (Fig. 1b) with an EC_{50} (concn. causing half-maximal effect) for PMA of 2.6 ± 0.59 nM. This increase in DG level occurred despite the inability of PMA to increase inositol phosphate accumulation (Brown *et al.*, 1987; Cook & Wakelam, 1991). For example, a 20 min incubation of [^3H]inositol-labelled cells in the presence of 10 mM-LiCl resulted in the following agonist-stimulated total inositol phosphate accumulation: control, 8417 ± 1583 d.p.m.; bombesin, 10514 ± 2322 d.p.m.; PMA, 6927 ± 64 d.p.m.

To assess if PMA-stimulated DG accumulation might be derived from the hydrolysis of a non-inositide lipid, experiments were performed on cells labelled for 48 h with [^3H]palmitic acid. Under these conditions the majority of [^3H]palmitate incorporated into phospholipids was found in PtdCho (50 %), with less than 3 % incorporated into PtdIns (results not shown). This protocol therefore allowed preferential labelling of the non-inositol-containing lipids. Stimulation of [^3H]palmitate-labelled Swiss 3T3 cells with 100 nM-PMA resulted in a time-dependent increase in total [^3H]DG which was apparent after 1 min but again was not significant until after a 2 min stimulation

Fig. 1. PMA-stimulated DG formation

PMA-stimulated changes in DG were determined in quiescent confluent cells as described in the Materials and methods section. (a) Time course of DG increase, measured in response to 100 nM-PMA; (b) dose-dependence of PMA-stimulated changes in DG mass; (c) PMA (100 nM)-stimulated increases in [3H]DG in cells prelabelled with [3H]palmitate. □, Control cells; ■, PMA-stimulated cells.

($P = 0.009$) (Fig. 1c). [3H]DG was maintained above control for 15 min (Fig. 1c) and up to 30 min (results not shown).

PMA stimulates the release of [3H]Cho before [3H]ChoP in [3H]Cho-labelled Swiss 3T3 cells (Cook & Wakelam, 1989), suggesting that PtdCho is initially hydrolysed by a PLD-like activity. To confirm activation of PLD, the ability of PMA-stimulated [3H]palmitate-labelled Swiss 3T3 cells to form [3H]-PtdBut in the presence of butan-1-ol was examined. This reaction is catalysed by the phosphatidyltransferase activity of PLD and serves as a sensitive unequivocal marker for PLD activity in whole cells, since phosphatidylalcohols are not formed by alternative pathways in whole cells (Pai *et al.*, 1988a). Stimulation of [3H]palmitate-labelled Swiss 3T3 cells with 100 nM-PMA in the presence of increasing concentrations of butan-1-ol resulted

Fig. 2. PMA-stimulated PLD activity

[3H]Palmitate-labelled cells were stimulated (a) with 100 nM-PMA for increasing times or (b) with increasing concentrations of PMA for 20 min in the presence of 30 mM-butan-1-ol, and the generation of PtdBut was determined as described in the Materials and methods section. ■, PMA; □, control. Results are means $\pm$ s.d. from a single typical experiment.

in the dose-dependent accumulation of a radioactive lipid which co-migrated exactly with [14C]PtdBut upon t.l.c. (results not shown). In addition, both PMA and bombesin stimulated the formation of [3H]PtdBut in unlabelled Swiss 3T3 cells stimulated in the presence of [3H]butan-1-ol as described by Randall *et al.* (1990) (results not shown). In [3H]palmitate-labelled cells maximum [3H]PtdBut formation was attained at 30 mM-butan-1-ol (0.3, v/v), whereas concentrations of the alcohol greater than 100 mM resulted in a decrease in [3H]PtdBut formation and cell lysis (results not shown). All experiments were therefore performed at 0.3% butan-1-ol, which was without any deleterious effect upon the cells during the exposure times reported here.

Stimulation of [3H]palmitate-labelled Swiss 3T3 cells with 100 nM-PMA in the presence of 0.3% butan-1-ol resulted in the time-dependent accumulation of [3H]PtdBut which was elevated above control after 1 min ($P < 0.01$) and rose in a near-linear, non-desensitizing manner for 30 min (Fig. 2a). In no experiment did the rate of PMA-stimulated [3H]PtdBut formation decrease over 30 min. The control [β -phorbol in 0.01% (v/v) dimethyl sulphoxide] did not increase [3H]PtdBut levels significantly over 30 min. The ability of PMA to stimulate [3H]PtdBut formation was dose-dependent (Fig. 2b) with half-maximal stimulation (EC_{50}) occurring at 2.8 ± 1.02 nM (mean $\pm$ s.d., $n = 3$ experiments in triplicate).

Since bombesin can also stimulate sustained increases in DG mass and release of [3H]Cho, experiments were performed to

Fig. 3. Bombesin-stimulated PLD activity

[^3H]Palmitate-labelled cells were stimulated (a) with 100 nM-bombesin for increasing times or (b) with increasing concentrations of bombesin for 20 min in the presence of 30 mM-butan-1-ol, and the generation of PtdBut was determined as described in the Materials and methods section. ■, Bombesin; □, Control. Results are means $\pm$ S.D. from a single typical experiment.

Table 1. Effect of butanol upon bombesin- and PMA-stimulated DG formation

Confluent and quiescent cells were washed in DMBS for 60 min, preincubated in DMBS containing 0.3% (v/v) butanol where stated for 5 min and then stimulated with 100 nM-bombesin or 100 nM-PMA for 15 min. DG formation was determined as described in the Materials and methods section. The results are means $\pm$ S.D. where $n = 3$ and are from a single experiment typical of three.

Stimulation	DG formation (pmol/ 10^6 cells)		Inhibition (%)
	Control	+ Butanol	
None	206 $\pm$ 13	250 $\pm$ 26	—
Bombesin	719 $\pm$ 12	526 $\pm$ 28	30 $\pm$ 5
PMA	625 $\pm$ 55	249 $\pm$ 15	74 $\pm$ 15

determine if bombesin could also stimulate [^3H]PtdBut formation in Swiss 3T3 cells. Fig. 3(a) shows that, in the presence of 0.3% (v/v) butan-1-ol, bombesin increased [^3H]PtdBut levels, significant increases were apparent at 15 and 30 s ($P < 0.01$) and levels rose up to 2 min, after which the rate of increase was greatly decreased but continued in some experiments up to 15 min; [^3H]PtdBut levels remained unchanged in the absence of stimuli. The dose-response curve for bombesin-stimulated [^3H]-

Fig. 4. Bombesin-stimulated inositol phosphate generation

[^3H]Inositol-labelled cells were stimulated with 100 nM-bombesin for the stated times in the presence of 10 mM-LiCl, and inositol phosphate accumulation was determined as described in the Materials and methods section. The results are means $\pm$ S.D. where $n = 3$ and the data are from a single typical experiment. ■, Bombesin; □, control.

PtdBut formation is shown in Fig. 3(b); the EC_{50} value was 1.3 ± 0.31 nM (mean $\pm$ S.D., $n = 3$ experiments in triplicate).

Since bombesin- and PMA-stimulated [^3H]PtdBut formation occurred prior to the sustained increase in DG mass for each agonist (Fig. 1a; Cook *et al.*, 1990) it seemed possible that the normal product of PLD, phosphatidic acid, might serve as the precursor of DG by the action of PPH. To assess this, butan-1-ol was used to 'trap' phosphatidate moieties such as PtdBut, which is a poor substrate for PPH (Bonser *et al.*, 1989; Metz & Dunlop, 1991). PMA-stimulated DG formation was inhibited by $74 \pm 14\%$ in the presence of 0.3% butan-1-ol, whereas bombesin-stimulated DG formation was only inhibited by $30 \pm 5\%$ (Table 1), suggesting different roles for a coupled PLD/PPH pathway in contributing to DG formation by the two agonists.

In addition to the hydrolysis of PtdCho, some reports have suggested that a slower, but sustained, hydrolysis of PtdIns or PtdIns(4,5) P_2 may contribute to sustained DG formation (Imai & Gershengorn, 1986; Griendling *et al.*, 1986). We have previously shown that bombesin-stimulated Ins(1,4,5) P_3 accumulation is complete by 30 s, and it quickly returns to pre-stimulated levels. Although the decrease in Ins(1,4,5) P_3 mass could imply a desensitization of PtdIns(4,5) P_2 hydrolysis, it could equally reflect an increased rate of metabolism of the second messenger. Studies in [^3H]inositol-labelled cells, performed in the presence of 10 mM-LiCl, showed that the accumulation of total inositol phosphates ([^3H]InsP) was markedly biphasic (Fig. 4). Significant increases in [^3H]InsP were observed after a 30 s stimulation with bombesin ($P = 0.001$), and from 0 to 1 min stimulation the rate of [^3H]InsP accumulation was typically 8-fold greater than the rate from 1–15 min or at 30 min. This suggested that the hydrolysis of inositol lipids was indeed desensitized, but that this desensitization was not complete, as indicated by the continued accumulation of inositol phosphates for up to 30 min (Fig. 4) and 1 h (results not shown).

We have previously shown that the release of [^3H]Cho from Swiss 3T3 cells stimulated with bombesin or PMA is abolished in cells depleted of cellular PKC activity (Cook & Wakelam, 1989, 1991). The results in Table 2 show that the ability of bombesin

Table 2. Effect of PKC down-regulation upon stimulated PLD activity

Swiss 3T3 cells were pretreated for 48 h with either 400 nM- β -phorbol or 400 nM-PMA. PtdBut formation was determined as described in the Materials and methods section in the presence of 0.3 % (v/v) butanol following a 20 min stimulation. The results are means $\pm$ S.D. where $n = 3$ from a single typical experiment. Because the incubation with PMA had a significant effect upon PtdCho labelling, the results have been normalized to the d.p.m. in the lipid fraction.

Stimulant	PtdBut (d.p.m.)	
	β -Phorbol-treated	PMA-treated
None	1654 $\pm$ 464	2866 $\pm$ 805
PMA	21695 $\pm$ 953	2224 $\pm$ 757
Bombesin	9864 $\pm$ 640	2427 $\pm$ 309

Fig. 5. Effect of Ro-31-8220 upon stimulated PLD activity

Cells (prelabelled with [3 H]palmitate) were preincubated with increasing concentrations of Ro-31-8220 for the stated times (■); control cells (□) received the vehicle (0.3 % dimethyl sulphoxide), for 5 min. The generation of PtdBut in response to a 20 min stimulation with 100 nM-PMA (a) or 100 nM-bombesin (b) was determined as described in the Materials and methods section.

and PMA to stimulate increases in [3 H]PtdBut was also completely abolished in down-regulated cells as compared with cells pre-treated with the vehicle control (β -phorbol in 0.1 % dimethyl sulphoxide).

Studies were also performed with the PKC inhibitor Ro-31-8220 (Davis *et al.*, 1989). Pre-treatment of [3 H]palmitate-labelled Swiss 3T3 cells with increasing doses of Ro-31-8220 for 5 min

Table 3. Effects of agents which elevate intracellular cyclic AMP upon bombesin-stimulated PLD activity

[3 H]Palmitate-labelled cells were pre-incubated with 10 μ M-forskolin, 0.1 mM-IBMX or 1 mM-dibutyryl cyclic AMP as stated for the appropriate times. The cells were then stimulated with bombesin in the presence of 0.3 % (v/v) butanol for 5 min and the generation of PtdBut was determined as described in the Materials and methods section. The results are means $\pm$ S.D. where $n = 3$ and are from a single experiment representative of three.

[Bombesin] (nM)	PtdBut (d.p.m.)	
	Control cells	Pre-treated cells
(a) Pre-treatment with forskolin and IBMX for 5 min		
1	1558 $\pm$ 300	1629 $\pm$ 99
10	2231 $\pm$ 264	2394 $\pm$ 289
(b) Pre-treatment with forskolin and IBMX for 10 min		
0	772 $\pm$ 226	1142 $\pm$ 104
1	1882 $\pm$ 265	1610 $\pm$ 305
10	2022 $\pm$ 124	2338 $\pm$ 39
(c) Pretreatment with dibutyryl cyclic AMP and IBMX for 15 min		
0	589 $\pm$ 115	620 $\pm$ 107
10	2296 $\pm$ 749	1687 $\pm$ 366

resulted in a dose-dependent inhibition of PMA-stimulated [3 H]PtdBut formation (Fig. 5a), confirming the result in the PKC-depleted Swiss 3T3 cells. Maximal inhibition, 92 ± 7 %, was attained at 10 μ M. In contrast, Ro-31-8220 only inhibited 45 ± 5 % of bombesin-stimulated [3 H]PtdBut formation at 10 μ M (Fig. 5b). Thus Ro-31-8220 inhibited essentially all of the PMA-stimulated PLD activity with an IC_{50} value (concn. causing half-maximal inhibition) of 0.87 ± 0.43 μ M, whereas the response to bombesin was inhibited by 50 % at most; an accurate IC_{50} for the bombesin response could not be determined, since the inhibition was not complete. These experiments were performed together with the same stock preparation of Ro-31-8220, so the differences between bombesin and PMA cannot be explained by variations in the preparation or efficacy of the inhibitor. Increasing the Ro-31-8220 concentration to 100 μ M permitted complete inhibition of bombesin-stimulated PLD activity; however, at these concentrations the effect of the inhibitor become non-specific (Davis *et al.*, 1989).

Tyagi *et al.* (1991) have shown that elevation of cyclic AMP inhibits fMet-Leu-Phe-stimulated PLD activity at a site proximal to PLD itself, raising the possibility of 'cross-talk' between the adenylate cyclase and PLD signal transduction pathways. In Swiss 3T3 cells, pre-treatment with 10 μ M-forskolin and 100 μ M-isobutylmethylxanthine (IBMX) for 5 or 10 min or 1 mM-dibutyryl cyclic AMP for 15 min had no effect upon subsequent bombesin-stimulated PLD activity (Table 3).

In addition to activation of PKC, an early response to stimulation of Swiss 3T3 cells with bombesin is an increase in [Ca^{2+}]_i, initially by mobilizing internal stores and later by entry of Ca^{2+} from outside the cell. To investigate whether the ion might play some role in the activation of PLD by bombesin, 2.5 mM-EGTA was included in the pre-incubation and stimulation DMBH buffer, to lower the external [Ca^{2+}] to 150 nM. Stimulation of Swiss 3T3 cells under these conditions resulted in a 50 % inhibition of bombesin-stimulated [3 H]PtdBut formation, suggesting that entry of Ca^{2+} was playing a role in activation of PLD by bombesin; the response to PMA was unaffected (Table 4). However, when Swiss 3T3 cells pre-incubated in normal or low- Ca^{2+} DMBH were pre-treated with Ro-31-8220 for 5 min

Table 4. Effect of EGTA upon agonist- and ionophore-stimulated PLD activity

Control cells or cells incubated in a buffer containing 2.5 mM-EGTA for 15 min in order to decrease the extracellular Ca^{2+} concentration to 150 nM were stimulated with 100 nM-bombesin, 100 nM-PMA or 5 μM -A23187 for 20 min and the generation of PtdBut was determined as described in the Materials and methods section. Ro-31-8220 was present at a concentration of 10 μM from 5 min prior to stimulation.

Stimulation	PtdBut (d.p.m.)	
	Control cells	EGTA-treated cells
None	938 $\pm$ 64	862 $\pm$ 428
Bombesin	13718 $\pm$ 170	6678 $\pm$ 289
Bombesin + Ro-31-8220	6468 $\pm$ 1105	6678 $\pm$ 289
PMA	17055 $\pm$ 267	15724 $\pm$ 470
A23187	4002 $\pm$ 319	2782 $\pm$ 297
A23187 + Ro-31-8220	1924 $\pm$ 283	1850 $\pm$ 49

Fig. 6. Effect of thapsigargin upon bombesin-stimulated PLD activity

Bombesin-stimulated PtdBut formation was determined following a 1 min stimulation with the stated bombesin concentration in the presence (■) or absence (□) of 1 μM -thapsigargin (the control cells received 0.1 % dimethyl sulphoxide as a vehicle control) as described in the Materials and methods section. The results are means $\pm$ S.D. where $n = 3$ and are from one experiment typical of three.

prior to addition of bombesin, the effects of EGTA and Ro-31-8220 were less than additive in terms of inhibition of bombesin-stimulated [^3H]PtdBut formation (Table 4). Thus, even in the presence of EGTA and Ro-31-8220, there was still residual bombesin-stimulated PLD activity, amounting to as much as 30 % of the maximum response. These results suggested that loss of increases in $[\text{Ca}^{2+}]_i$ (presumably by preventing Ca^{2+} entry) and inhibition of PKC might be exerting their effects at the same site in terms of PLD activity.

To determine if activation of PLD could arise simply by increasing the $[\text{Ca}^{2+}]_i$, the ability of the calcium ionophore A23187 to stimulate [^3H]PtdBut formation was examined. Addition of 5 μM -A23187 to [^3H]palmitate-labelled Swiss 3T3 cells in the presence of 0.3 % (v/v) butan-1-ol resulted in the formation

of [^3H]PtdBut (Table 4), although the response was clearly smaller than that caused by bombesin or PMA. However, when cells were pre-treated with Ro-31-8220 for 5 min before addition of A23187, the response was inhibited by at least 50 % (Table 4), suggesting that much of the effect of Ca^{2+} entry upon PLD activity was mediated by activation of PKC. Pre-treatment with EGTA inhibited A23187-stimulated [^3H]PtdBut formation, but only by 50 %.

Agonists also elevate $[\text{Ca}^{2+}]_i$ by stimulating the release of Ca^{2+} from the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive intracellular store. This store can be emptied by the addition of the tumour promoter thapsigargin, an inhibitor of the Ca^{2+} -ATPase (Thastrup *et al.*, 1990). At doses ranging from 10 nM to 10 μM , thapsigargin was unable to stimulate PLD activity during a 90 s stimulus (results not shown) and was without effect upon the dose-dependency of bombesin-stimulated PtdBut formation (Fig. 6). Longer incubation times were not employed because of the possibility of thapsigargin stimulating Ca^{2+} entry (Takemura *et al.*, 1989).

DISCUSSION

The results in Fig. 1(a) show that addition of PMA to quiescent Swiss 3T3 cells stimulated the marked accumulation of DG mass which was apparent from 1 min onwards and was maintained for at least 60 min (results not shown). In [^3H]palmitate-labelled Swiss 3T3 cells, where PtdCho was the major and PtdIns the minor labelled phospholipid, PMA also stimulated an increase in total [^3H]DG (Fig. 1c) with similar kinetics to the response in Fig. 1(a). Taken together with the inability of PMA to stimulate increases the inositol phosphate accumulation, this suggests that PMA was able to stimulate the hydrolysis of a non-inositide phospholipid which served as the source of DG; the observation that PMA also stimulates the release of [^3H]Cho and [^3H]ChoP (Cook & Wakelam, 1989; Price *et al.*, 1989) suggests that this lipid is PtdCho. In addition, the EC_{50} for PMA-stimulated DG formation was essentially the same as that for PMA-mediated inhibition of bombesin-stimulated inositol phosphate accumulation of Swiss 3T3 cells (Brown *et al.*, 1987; Cook & Wakelam, 1991). Thus PMA effectively dissociated DG accumulation from inositol lipid hydrolysis.

These results confirm those of Takuwa *et al.* (1987b), but we have sought to advance our studies by defining the primary route of PtdCho hydrolysis, since the former study had not addressed this issue. We have previously identified Cho as the primary product of PtdCho hydrolysis, suggesting activation of a PLD (Cook & Wakelam, 1989; Price *et al.*, 1989) suggests that this lipid is PtdCho. In addition, the EC_{50} for PMA-stimulated DG and ChoP. The results in Fig. 2(a) confirm that the release of [^3H]Cho was indeed due to activation of PLD, since in [^3H]palmitate-labelled cells PMA stimulated the formation of [^3H]PtdBut in the presence of butan-1-ol by the transferase activity unique to PLD. The demonstration that PMA-stimulated PLD activity was maintained, unabated, for at least 30 min is consistent with it serving as a pathway for sustained DG formation, and indeed the EC_{50} for [^3H]PtdBut formation (Fig. 2b) was the same as that for DG formation (Fig. 1c) and activation of PKC (Castagna *et al.*, 1982).

Bombesin also stimulated PLD activity as judged by the formation of [^3H]PtdBut, and the kinetics of this response was essentially identical to those for the release of [^3H]Cho in isotopically labelled cells (Cook & Wakelam, 1989). However, in contrast to the results with PMA, [^3H]PtdBut formation was apparently rapidly desensitized, such that the rate from 2 min onwards to 15 min was up to 10-fold lower than the initial rate over the first 2 min of the time course. The EC_{50} for bombesin-stimulated PLD activity (Fig. 3b) was essentially the same as that

for bombesin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ mass formation and total inositol phosphate accumulation (Cook *et al.*, 1990; Cook & Wakelam, 1991), suggesting that activation of PLC and PLD was mediated through the same population of receptors.

Since $[^3\text{H}]\text{PtdBut}$ formation preceded sustained DG formation, this was consistent with the phosphatidic acid produced by PLD serving as a precursor for DG by a coupled PLD/PPH pathway as described for neutrophils (Bonser *et al.*, 1989). The results in Table 1 show that this may well be the case for PMA, since butan-1-ol inhibited 70 % of PMA-stimulated DG formation, suggesting a major role for a coupled PLD/PPH pathway in PMA-stimulated DG formation. In contrast, bombesin-stimulated DG formation was only inhibited by 30 % in the presence of butan-1-ol, suggesting a minor role for a PLD/PPH pathway in bombesin-stimulated DG formation (Table 1). It is unlikely that these differences are due to there being incomplete competition between butan-1-ol and water for accepting the released phosphatidyl moiety in the PLD reaction, since $[^3\text{H}]\text{PtdBut}$ formation was clearly maximal for both PMA and bombesin at 0.3 % (v/v) butan-1-ol, and the EC_{50} for butan-1-ol-dependent $[^3\text{H}]\text{PtdBut}$ formation was similar for both agonists (8.95 ± 1.11 mM for PMA and 3.75 ± 0.33 mM for bombesin). Rather, it seems likely that the differences in the role of PLD in DG formation may reflect the differences in duration of activation of PLD by the two agonists. Since $[^3\text{H}]\text{PtdBut}$ is poorly metabolized by cells (Bonser *et al.*, 1989; Metz & Dunlop, 1991) it accumulates and serves as an index for PLD activity. PMA-stimulated PLD activity was sustained for at least 30 min and may therefore normally provide a sustained source of phosphatidic acid, and therefore DG, by the PLD/PPH pathway. In contrast, bombesin-stimulated PLD activity was rapidly desensitized, such that PLD activity had virtually ceased after 5 min. Since bombesin-stimulated DG was elevated for at least 30 min, this suggests either that PLD plays only a transient role in DG accumulation over the first 5 min of the response or that the phosphatidic acid produced by PLD over 5 min is slowly converted to DG to account for the sustained accumulation as recently suggested (Martinson *et al.*, 1989). This latter explanation seems unlikely, since PPH activity appears not to play a rate-limiting role in the coupled PLD/PPH pathway of DG formation (Martin, 1988). Thus it seems that bombesin-stimulated PLD activity plays only a transient role in the second phase of bombesin-stimulated DG formation. Alternative pathways could involve PtdCho-PLC activity, since bombesin is also able to stimulate the release of $[^3\text{H}]\text{ChoP}$ (Cook & Wakelam, 1989; Price *et al.*, 1989), or the continued hydrolysis of inositol lipids. In support of the latter possibility, bombesin-stimulated accumulation of $[^3\text{H}]\text{InsP}$ and total $[^3\text{H}]\text{inositol}$ phosphates continues, albeit at a reduced rate, for at least 30–60 min after $\text{Ins}(1,4,5)\text{P}_3$ levels have declined to pre-stimulated levels (Fig. 4). Whether this represents sustained PtdIns hydrolysis (Imai & Gershengorn, 1986), or continued but slower $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis, remains to be determined.

Chronic treatment of Swiss 3T3 cells with 400 nM-PMA to down-regulate PKC (Rodriguez-Pena & Rozengurt, 1984) resulted in the complete loss of bombesin- and PMA-stimulated PLD activity (Table 2), suggesting that the response was absolutely dependent upon prior inositol lipid hydrolysis and activation of PKC. This confirms previous results where bombesin- and PMA-stimulated $[^3\text{H}]\text{Cho}$ release was abolished under similar conditions (Cook & Wakelam, 1989, 1991; Price *et al.*, 1989). In contrast, there were marked differences in the ability of the PKC inhibitor, Ro-31-8220, to inhibit agonist-stimulated PLD activity; the loss of 92 ± 7 % of PMA-stimulated PLD activity was consistent with the result in PKC-depleted cells, suggesting that PMA exerts its effect on PLD via activation of

PKC. However, bombesin-stimulated PLD activity was inhibited by at most 50 % at the same concentration at which the PMA response was maximally inhibited, suggesting an additional PKC-independent mechanism. Higher concentrations of Ro-31-8220 (100 μM) allowed complete inhibition of the bombesin response, but this cannot be attributed to selective effects upon PKC; Ro-31-8220 acts at the ATP-binding site and so at these concentrations will act as non-selective kinase inhibitor (Davis *et al.*, 1989). Bombesin-stimulated PLD activity may be mediated by a distinct PKC isoform which is sensitive to down-regulation but relatively less sensitive to Ro-31-8220. Nevertheless, there remains the intriguing anomaly that down-regulation abolishes all bombesin-stimulated PLD activity; whether this suggests that PLD may also been down-regulated by prolonged PMA treatment as discussed by Billah & Anthes (1990) is unclear, but it does suggest that experiments with PKC-depleted cells should be confirmed with PKC inhibitors.

The finding that agents which elevate intracellular cyclic AMP (forskolin, IBMX and dibutyryl cyclic AMP) did not inhibit bombesin-stimulated PLD activity (Table 3) is in contrast to the report of Tyagi *et al.* (1991), which showed that PKA exerted an effect upstream of PLD in neutrophils. In some experiments a small potentiation of the response was observed, but this was not always statistically significant (results not shown). This implies that cross-talk involving adenylate cyclase/PKA is not a major pathway for regulation of PLD in Swiss 3T3 cells. This may reflect a tissue- or species-specific distribution of components in these two signal transduction pathways.

Since an increased $[\text{Ca}^{2+}]_i$ is also an early signal resulting from bombesin-stimulated $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis, experiments were performed with thapsigargin, A23187 and with EGTA to buffer extracellular $[\text{Ca}^{2+}]$ to 150 nM, the approximate resting $[\text{Ca}^{2+}]_i$ in Swiss 3T3 cells (Hesketh *et al.*, 1988). The major difficulty in interpreting chelator experiments is that it cannot be assumed that the sole effect of EGTA treatment is to prevent Ca^{2+} entry; there may be depletion of intracellular stores during the treatment period. In addition, Ca^{2+} may not play a dominant, stimulatory role in PLD activity, but may function as a vital co-factor, depletion of which during EGTA treatment may modulate PLD activity. EGTA treatment had no effect upon PMA-stimulated PLD activity (Table 3), consistent with PMA exerting its effect solely by activation of PKC. EGTA treatment alone inhibited bombesin-stimulated PLD activity by some 50 %, suggesting that increases in $[\text{Ca}^{2+}]_i$ are involved in activation of PLD. However, when EGTA treatment was combined with the PKC inhibitor Ro-31-8220, the inhibition of PLD activity was less than additive, suggesting that Ro-31-8220 and EGTA were exerting some of their effects at the same site.

Addition of A23187 to Swiss 3T3 cells resulted in the activation of PLD activity and this was inhibited, though not completely, by chelation of extracellular Ca^{2+} . Thus increases in $[\text{Ca}^{2+}]_i$ are able to activate PLD. However, pre-treatment with 10 μM -Ro-31-8220 inhibited 50 % of A23187-stimulated PLD activity, suggesting that at least half of this response was perhaps due to Ca^{2+} acting as a co-factor in activation of PKC. This may explain why the inhibition of bombesin-stimulated PLD activity by EGTA and Ro-31-8220 was not completely additive, since at least some of the effects of increased $[\text{Ca}^{2+}]_i$ can be attributed to activation of PKC.

The remaining 30 % of bombesin-stimulated PLD activity which persisted even after treatment with Ro-31-8220 and EGTA may represent regulation of PLD by mobilization of intracellular stored Ca^{2+} or perhaps by DG itself; DG has been reported to stimulate PLA_2 activity *in vitro* at the level of the enzyme rather than via activation of PKC (Burch, 1988). Elevation of $[\text{Ca}^{2+}]_i$ by treatment with the endoplasmic reticulum Ca^{2+} -ATPase inhibitor

thapsigargin failed to stimulate PLD activity. Additionally, since the agent was without effect on the EC_{50} for bombesin-stimulated [3H]PtdBut formation (Fig. 5), it can be concluded that thapsigargin-stimulated Ca^{2+} release from the $Ins(1,4,5)P_3$ -sensitive store is insufficient to activate PLD. Despite these results, we cannot rule out a role for Ca^{2+} mobilized from the $Ins(1,4,5)P_3$ -sensitive store in the regulation of PLD activity, because thapsigargin elicits a relatively small increase in $[Ca^{2+}]_i$ (Takemura *et al.*, 1989; Thastrup *et al.*, 1990) in comparison to bombesin (Takuwa *et al.*, 1987b). Nonetheless, since A23187-stimulated PLD activity was partially inhibited by Ro-31-8220, it appears that the effect of increased $[Ca^{2+}]_i$ is subordinate to PKC and not solely due to direct activation of PLD.

In summary, we have shown that PMA stimulates a sustained increase in PLD activity and DG mass in Swiss 3T3 cells. Whereas PMA-stimulated DG formation appears to be derived by a PLD/PPH pathway, since it is largely inhibited by butan-1-ol, bombesin-stimulated PLD activity appears to be a transient response, making only a minor contribution to sustained DG formation. The kinetics of PMA-stimulated DG formation appear to mimic those of the sustained response to bombesin; however, in mechanistic terms the two responses are quite different. The large contribution made to PMA-stimulated DG formation by PLD probably reflects the persistent nature of phorbol ester activation of PKC. The activation of PLD by bombesin appears to be determined largely by the two major signals derived from PtdIns(4,5) P_2 hydrolysis, increased $[Ca^{2+}]_i$ and PKC activation, with no evidence for regulation by PKA. The sustained phase of bombesin-stimulated DG formation may be a composite of a number of pathways, including transient PLD activation, a potential PtdCho-specific PLC pathway and sustained inositol lipid hydrolysis.

We thank R. Plevin and A. Paterson for advice on the DG kinase assay and helpful discussions, Roche U.K. for the provision of Ro-31-8220 and Wellcome p.l.c. (Beckenham, Kent, U.K.) for provision of a [3H]PtdBut standard. This work was supported by grants from the Cancer Research Campaign and the Wellcome Trust. S. J. C. was in receipt of an S.E.R.C.-CASE studentship with Wellcome p.l.c., and C. P. B. is a Wellcome Prize Student.

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Received 28 May 1991; accepted 12 July 1991