The roles of multiple pathways in regulating bombesin-stimulated phospholipase D activity in Swiss 3T3 fibroblasts

Celia P. BRISCOE,†‡ Ashley MARTIN,* Michael CROSS*† and Michael J. O. WAKELAM*†§

*University of Birmingham Institute for Cancer Studies, Clinical Research Block, Queen Elizabeth Hospital, Birmingham B15 2TH, U.K., and †Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

The regulation of bombesin-stimulated phospholipase D (PLD) activity in Swiss 3T3 fibroblasts was examined. Increasing protein-tyrosine phosphorylation by using pervanadate to inhibit tyrosine phosphatases was found to stimulate protein kinase C (PKC)-independent [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) accumulation within 5 min, which continued to increase up to 30 min. The stimulation of PLD activity in response to submaximal [bombesin] could be decreased by approx. 50% by the tyrosine kinase inhibitor genistein, whereas pretreatment with genistein and the PKC inhibitor Ro-31-8220 completely abolished the generation of [^{3}H]PtdBut in response to a maximal concentration of bombesin. The addition of guanosine 5′-[γ-thio]triphosphate (GTP[S]) into permeabilized cells resulted in an increase in [^{3}H]PtdBut, which was abolished by depletion of cellular ATP. The additional presence of 30 μM GTP[S] did not increase the stimulation of PLD activity by any [bombesin] tested, whereas it was synergistic with that stimulated in response to phorbol 12-myristate 13-acetate. These findings suggest that bombesin-stimulated PLD activity is indirectly regulated by G-proteins, possibly through a kinase intermediate. Furthermore, activation of protein tyrosine kinases is proposed to account for the PKC-independent arm of bombesin-stimulated PLD activity. No evidence was obtained for a form of PLD directly regulated by tyrosine phosphorylation.

INTRODUCTION

Bombesin exemplifies an extensive range of hormones and growth factors which have been demonstrated to activate the phospholipase D (PLD)-catalysed hydrolysis of phosphatidylcholine (PtdCho) to generate choline and phosphatidic acid (PtdOH) [1,2]. The peptide is a mitogen for Swiss 3T3 cells [3], binds to a single class of high-affinity cell-surface receptors [4] and also stimulates phospholipase C (PLC)-mediated PtdIns(4,5)P_2 hydrolysis generating the second-messenger molecules Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol. Ins(1,4,5)P_3 stimulates a transient increase in $[Ca^{2+}]_i$ [5], while diacylglycerol activates protein kinase C (PKC) [6].

It has been suggested that PKC activation stimulates PLD activity, and previously we have demonstrated that bombesin-stimulated PLD activity can be inhibited by treatment of cells with the selective PKC inhibitor Ro-31-8220, but by only approx. 50% [7]. It was possible that the PKC-insensitive component of PLD activation involved an elevation of intracellular $[Ca^{2+}]$; however, experiments where $[Ca^{2+}]$ was elevated with thapsigargin, where external $[Ca^{2+}]$ was depleted with EGTA and where Ca^{2+} entry was stimulated with A23187 suggested that the role of changes in $[Ca^{2+}]$ was minimal, and indeed appeared to be mediated through effects on PKC activity [7].

Two possible PKC-independent regulatory pathways of PLD activity are through tyrosine phosphorylation, particularly as bombesin stimulates the PKC-independent tyrosine phosphorylation of a number of proteins in Swiss 3T3 cells [8], or through the direct stimulation by a receptor-activated G-protein. The potential involvement of tyrosine kinases in the activation of PLD was realized by stimulation of the enzyme activity by epidermal growth factor [9]. This was found to occur independently of inositol lipid hydrolysis and PKC activation, but activation of the receptor kinase was essential. Furthermore, the stimulation of PLD activity through cytosolic tyrosine kinases has been reported in cells such as *N*-formylmethionyl-leucyl-phenylalanine-stimulated neutrophils [10] and endothelin-stimulated A10 vascular-smooth-muscle cells [11].

The regulation of PtdIns-PLC by guanine nucleotides is now well established [12]. Since many agonists that activate PLD also stimulate PLC-catalysed PtdIns(4,5)P_2 hydrolysis, the possible regulation through G-proteins has been investigated in several cell lines. The addition of non-hydrolysable GTP analogues such as guanosine 5′-[γ-thio]triphosphate (GTP[S]) to permeabilized cells [13,14] and cell membranes [15,16] has shown that receptors can regulate PLD activity through the activation of G-proteins. The use of cell-free systems from neutrophils and granulocytes [17,18] established that stimulation of PLD activity with GTP[S] required factors in both the plasma membrane and the cytosol. It has been recently proposed that low-molecular-mass G-protein ADP-ribosylation factor (ARF) is responsible for such regulation [19,20]. However, the differential sensitivity of stimulated PLD activity to pertussis-toxin treatment suggested that different heterotrimeric G-proteins could regulate PLD activity, in an analogous manner to the G-protein-regulated PtdIns-PLC activity [12]. The bombesin receptor couples to PtdIns-PLC through the pertussis-toxin-insensitive G_q [21,22]. Therefore the possibility that bombesin may regulate PLD activity through a G-

Abbreviations used: ARF, ADP-ribosylation factor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; GDP[S], guanosine 5′-[β-thio]diphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; HHBG, Hepes-buffered Hanks' buffered salt solution containing 10 mM glucose and 1% (w/v) BSA; HHBG.Li, HHBG containing 10 mM LiCl; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid.

‡ Present address: Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0634, U.S.A.

§ To whom correspondence should be addressed, in Birmingham.

protein, perhaps through the same as, and/or a different one from, that coupled to the hydrolysis of PtdIns(4,5)P_2 was also investigated.

MATERIALS AND METHODS

Cell culture

Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) newborn-calf serum, 27 mg of glutamine/ml and penicillin/streptomycin (250 units/ml and 250 mg/ml respectively) at 37 °C in a humidified atmosphere of air/CO_2 (19:1). Cells were routinely passaged when subconfluent into 24-well plates. Cells were grown to confluency and quiescence by lowering the serum concentration to 2% (v/v) for 24 h before experiment. For labelling studies, the relevant radiochemical {[9,10(n)-^{3}H]palmitic acid, 4 μCi/ml; *myo*-[^{3}H]inositol, 1 μCi/ml} was included in the 2% serum-containing medium.

Assay of PLD transphosphatidylation activity in whole cells

Quiescent Swiss 3T3 cells labelled with [^{3}H]palmitic acid were washed in 0.5 ml of Hepes-buffered Hanks buffered saline solution, pH 7.4, containing 10 mM-glucose, 1% (w/v) BSA and 20 mM Hepes (HHBG), for 20 min at 37 °C before incubation for a further 5 min in 0.5 ml of HHBG containing 30 mM butan-1-ol. Incubations were commenced by replacing the medium with 0.2 ml of HHBG/butan-1-ol and the stimulant at the concentrations and times indicated. Incubations were terminated by removal of the medium and addition of ice-cold methanol. After extraction of lipids in chloroform, [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) was separated by t.l.c. as described previously [7].

Measurement of total inositol phosphates

Quiescent Swiss 3T3 cells grown in 24-well plates and labelled with [^{3}H]inositol were washed in 0.5 ml of HHBG for 2 × 10 min at 37 °C before incubation for a further 5 min in 0.5 ml of HHBG containing 10 mM LiCl (HHBG.Li). Incubations were commenced by replacing the medium with 200 μl (or 150 μl if reaction was to be terminated with methanol) of the test reagent in HHBG.Li, at the times and concentrations indicated. Incubations were terminated by addition of either 100 μl of 10% (v/v) $HClO_4$ or 500 μl of methanol, and the samples were extracted on ice. The formation of total inositol phosphates was determined by ion-exchange chromatography on Dowex 1 × 8 as described previously [2].

Determination of the generation of total inositol phosphates and PLD transferase activities in the same cell monolayer

Quiescent Swiss 3T3 cells labelled in 0.5 ml of inositol-free DMEM containing 2% dialysed newborn-calf serum and 1 μCi/ml *myo*-[2-^{3}H]inositol + 4 μCi/ml [^{3}H]palmitic acid were treated as described above for measurement of total inositol phosphates, by using methanol to terminate the reaction, except that both 30 mM butan-1-ol and 10 mM LiCl were included in the buffer. The samples were processed as described for the assay of total inositol phosphates, except that cell debris was transferred to screw-top glass vials. After splitting the phases, the whole of the aqueous phase was taken for total inositol phosphate measurement, while the organic phase was dried down and used for the analysis of [^{3}H]PtdBut generation as described above. Control experiments in cells labelled with [^{3}H]palmitic acid showed that the presence of 10 mM LiCl did not affect stimulated [^{3}H]PtdBut generation, nor did the presence of butan-1-ol in [^{3}H]inositol-labelled cells affect stimulated generation of total inositol phosphates (results not shown).

Measurement of PtdIns 3-kinase and PLD activities in immunoprecipitates

Swiss 3T3 cells were incubated with 0.5 mM pervanadate for 20 min and then lysed in 1% (w/v) Nonidet P40/10% (v/v) glycerol/20 mM Tris/HCl (pH 8)/137 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/0.5 mM Na_4VO_3, containing 10 μg/ml leupeptin and 0.2 mM phenylmethanesulphonyl fluoride. The lysates were centrifuged at 14000 *g* for 10 min at 4 °C, and the supernatant was transferred to a fresh tube; protein content was determined with the BCA kit (Pierce, U.K.). A solution containing 100 μg of protein was incubated with 2.5 μl of PY54 anti-phosphotyrosine antibody overnight at 4 °C, and 20 μl of 50% (v/v) Protein G–Sepharose (Sigma) was then added for 2 h at 4 °C. The immunoprecipitates were washed successively at 4 °C as follows: 2 × 1 ml of lysis buffer, 2 × 1 ml of 0.5 M LiCl/0.1 M Tris/HCl, pH 8.0, 1 × 1 ml of 0.1 M NaCl/1 mM EDTA/10 mM Tris/HCl, pH 7.6, and finally 1 × 1 ml of 5 mM $MgCl_2$/20 mM Hepes/NaOH, pH 7.4.

PtdIns 3-kinase activity in the immunoprecipitates was determined by the method of Jackson et al. [23]. PLD activity was determined by the method of Brown et al. [19], using a phosphatidylethanolamine/PtdInsP_2/PtdCho (molar proportions 16:1.4:1) micellar substrate where the [^{3}H]PtdCho concentration was 8.6 μM, with a specific radioactivity of 58 Ci/mol.

Cell permeabilization

Quiescent Swiss 3T3 cells grown in 24-well plates and labelled with the appropriate radioisotope were washed in 0.5 ml of HHBG for 20 min at 37 °C before addition for 5 min of streptolysin O (0.6 i.u./ml) in permeabilization buffer containing 20 mM Hepes, 120 mM KCl, 8.49 mM $MgCl_2$, 61 μM $CaCl_2$, 2 mM KH_2PO_4, 0.1 mM EGTA, 0.1% BSA and 2.5 mM ATP (free Ca^{2+} concn. 150 nM) as previously described [24]. This was followed by washing with 2 × 150 μl of permeabilization buffer over 10–15 s and stimulation with 150 μl of the test reagent in permeabilization buffer at the concentrations and times indicated. After termination of the incubation, permeabilized cells were treated exactly as described for the appropriate assay in intact cells. Permeabilization was determined to be maximal (approx. 90%) at a streptolysin O concentration of 0.6 i.u./ml by measuring the release of lactate dehydrogenase activity and the entry of the fluorescent dye bisbenzimide (results not shown).

In experiments where cells were depleted of cellular ATP, glucose was omitted from HHBG and the cells were also pretreated with the metabolic inhibitors 5 μM antimycin A and 6 mM 2-deoxyglucose for 4 min before permeabilization as described previously [24]. Permeabilization, subsequent washes and stimulation of cells were performed in permeabilization buffer lacking ATP but containing antimycin A and 2-deoxyglucose.

Western-blot analysis of tyrosine-phosphorylated proteins

Quiescent Swiss 3T3 cells were washed for 20 min in HHBG containing 0.1% BSA (w/v) at 37 °C, before treatment with the test reagent. Incubations were terminated by rapidly washing the cells three times in a wash buffer containing 50 mM Hepes, pH 7.4, 5 mM EGTA, 150 mM NaCl, 100 μM sodium orthovanadate and 200 μM phenylmethanesulphonyl fluoride. Lysis

buffer, containing 50 mM Hepes, pH 7.4, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 μg/ml aprotinin, 25 mM benzamidine, 5 μg/ml leupeptin and 100 μM sodium orthovanadate, was then added and the cells were placed on ice. The flasks or plates were shaken at 4 °C for 10–20 min after lysis and then the lysed cells were removed by scraping into Eppendorf tubes. The lysates were centrifuged at 4 °C at 14000 *g* for 10 min to remove the insoluble material, and the nuclei and the supernatant were transferred to fresh Eppendorf tubes. Lysates were stored at −20 °C until required.

Proteins were separated by SDS/PAGE, transferred to nitrocellulose, and those phosphorylated on tyrosine residues were detected with the anti-phosphotyrosine antibody PY54. Detection of antibody-bound proteins was with sheep anti-mouse IgG linked to horseradish peroxidase and ECL (Amersham International, Amersham, Bucks., U.K.). Luminescence was detected by exposing autoradiograph film to the blot for times ranging from 10 s to 5 min.

Expression of results

All data are from at least three separate experiments; unless otherwise stated, results are expressed as means ± S.D. Statistical analysis used an unpaired Student's *t* test.

Materials

Culture media, biochemicals and radioisotopes were from previously reported sources [7,24]. Antibodies were from Affiniti Research Products, Nottingham, U.K., and Sigma, Poole, Dorset, U.K. Ro 31-8220 [26] was generously given by Roche Pharmaceuticals.

RESULTS

Increasing protein tyrosine phosphorylation stimulates PLD activity

The analysis of protein tyrosine phosphorylation by immunoblotting of Swiss 3T3 cell lysates with an anti-phosphotyrosine monoclonal antibody demonstrated that bombesin stimulated a rapid increase in the phosphorylation of proteins corresponding to molecular masses of approx. 110–130 kDa, 80–85 kDa, 65–70 kDa and 40 kDa, which was detectable within 10 s and was maximal with a bombesin concentration of 0.3 nM (results not shown). This time course and dose–response were similar to those reported to Zachary et al. [8].

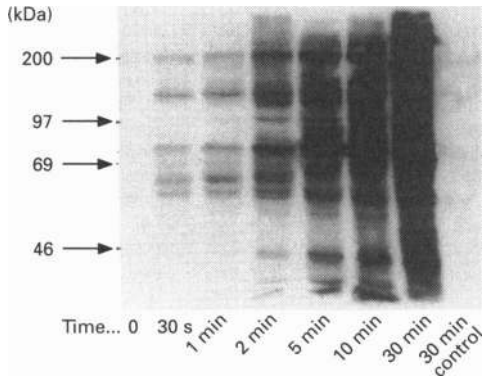

Figure 1 Time course of protein tyrosine phosphorylation in pervanadate-treated cells

Cells were stimulated with vehicle or 0.5 mM pervanadate for times as indicated. Cells were lysed and a sample, containing 30 μg of protein, was analysed for protein tyrosine phosphorylation as described. Results are from a single experiment representative of three. The values on the left indicate the positions of molecular mass markers.

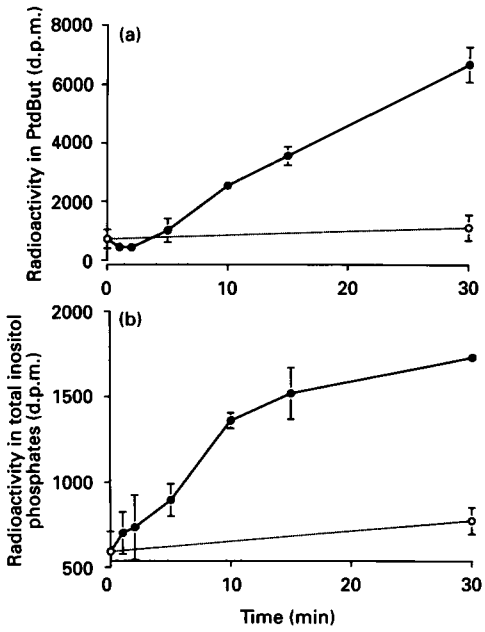

Figure 2 Time course of PLD activity and generation of total inositol phosphates in pervanadate-treated double-labelled cells

Cells labelled with [^{3}H]palmitate and [^{3}H]inositol were preincubated for 10 min with 10 mM LiCl and 30 mM butanol, then stimulated for the times indicated with 0.5 mM pervanadate (●) or vehicle (○). Chloroform extracts were prepared, and the generation of (**a**) [^{3}H]PtdBut and (**b**) total [^{3}H]inositol phosphates was quantified as described. Results are expressed as radioactivity in (**a**) [^{3}H]PtdBut or (**b**) total [^{3}H]inositol phosphates (d.p.m.; mean ± S.D., $n = 3$) and are from a single experiment representative of three.

Inhibition of phosphotyrosine phosphatase activity by pervanadate [27], which has been shown to stimulate tyrosine phosphorylation in a number of cells, including human platelets and T-cells [28,29], was used to investigate whether increasing tyrosine phosphorylation in the absence of a receptor-stimulation event could activate PLD. Figure 1 shows that 0.5 mM pervanadate stimulated rapid tyrosine phosphorylation in Swiss 3T3 cells, which was observable by 30 s and increased up to 30 min. The tyrosine phosphorylation of proteins with molecular masses of approx. 200 kDa, 110–130 kDa, 97 kDa, 80 kDa, 65 kDa and 45 kDa was observed.

In order to determine the effect of pervanadate on the activation of PLD and PtdIns-PLC, cells labelled with both [^{3}H]palmitate and [^{3}H]inositol were used. Figure 2 shows that pervanadate stimulated significant increases in both [^{3}H]PtdBut and total [^{3}H]inositol phosphates after 10 min, a time course that lagged behind that for protein tyrosine phosphorylation. Although the extent of stimulation varied considerably between experiments, the fold stimulation of the production of total [^{3}H]inositol phosphates over basal was generally less than that of [^{3}H]PtdBut.

Figure 3(a) shows that 10 μM of the selective PKC inhibitor Ro-31-8220 [26] had a negligible effect on protein tyrosine phosphorylation in cells stimulated with pervanadate for 10 min, but slightly decreased the phosphotyrosine content of proteins in cells stimulated with both 100 nM bombesin and pervanadate. Similar results were achieved with a 5 min stimulation with

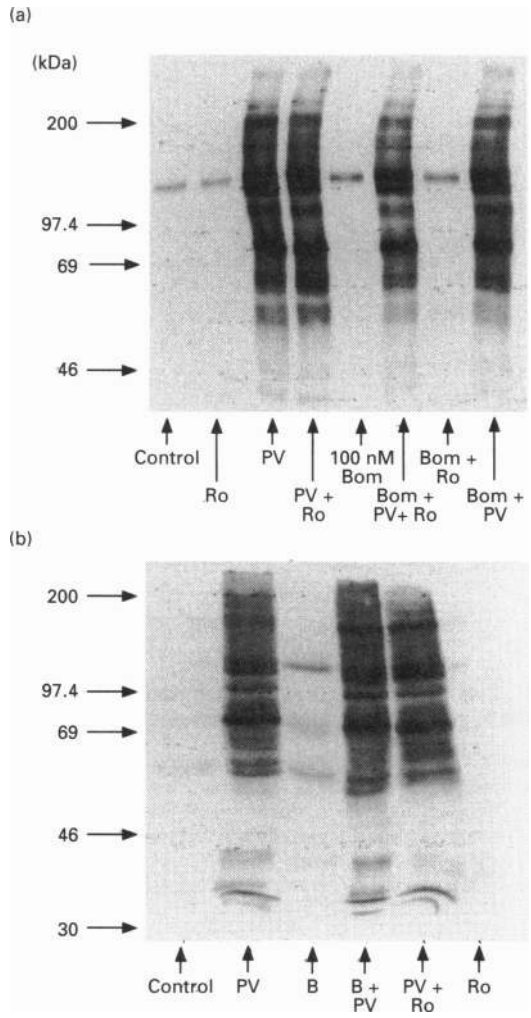

Figure 3 Effect of bombesin and Ro-31-8220 on protein tyrosine phosphorylation in pervanadate-treated cells

Cells were preincubated with vehicle (0.1% DMSO) or 10 μM Ro-31-8220 for 10 min, then stimulated in the presence of Ro-31-8220 (Ro) or vehicle (0.1% DMSO) as follows. (**a**) For 10 min with 0.5 mM pervanadate (PV), vehicle or 100 nM bombesin (Bom). Cells were lysed, and a sample of cell lysate corresponding to approx. 15 μg of protein was analysed for protein tyrosine phosphorylation as previously described. (**b**) For 5 min with 0.5 mM pervanadate (PV), vehicle or 3 nM bombesin (B). Cells were lysed, and a portion of cell lysate containing approx. 30 μg of protein was analysed for protein tyrosine phosphorylation as previously described. Results are from single experiments representative of three. The values on the left indicate the positions of molecular mass markers.

0.5 mM pervanadate and 3 nM bombesin (Figure 3b). In the experiment illustrated in Figure 3, the ECL exposure times were extremely short, in order that the phosphorylation induced by pervanadate could be clearly seen; therefore the definition of phosphorylated proteins in the bombesin-stimulated cells is poor. Despite its inhibitory effect on bombesin-stimulated PLD activity, 10 μM Ro-31-8220 did not attenuate the [^{3}H]PtdBut accumulation stimulated by a 10 min incubation with 0.5 mM pervanadate (Table 1). Experiments were also performed where 0.1 nM bombesin was used to activate PLD for 5 min in the presence of 0.5 mM pervanadate. Although tyrosine phosphorylation was elevated in response to pervanadate at this time point, there was no potentiation of bombesin-stimulated PLD activity (results not shown).

Table 1 Effect of bombesin and Ro-31-8220 on pervanadate-stimulated PLD activity

[^{3}H]Palmitate-labelled cells were preincubated with vehicle [0.1% dimethyl sulphoxide (DMSO)] or 10 μM Ro-31-8220 for 10 min in the presence of 30 mM butanol, then stimulated for 10 min in the absence or presence of 0.5 mM pervanadate, with vehicle or 100 nM bombesin. Incubations were terminated by addition of ice-cold methanol. Chloroform extracts were prepared, and the generation of [^{3}H]PtdBut was quantified as described in the text. Results are expressed as radioactivity in [^{3}H]PtdBut (mean $\pm$ S.D., $n = 3$) and are from a single experiment representative of three.

Treatment	Radioactivity in PtdBut (d.p.m.)
Control	1191 $\pm$ 418
Control + Ro-31-8220	1499 $\pm$ 67
Pervanadate	6356 $\pm$ 1104
Bombesin	6770 $\pm$ 612
Bombesin + Ro-31-8220	3428 $\pm$ 433
Pervanadate + Ro-32-8220	5162 $\pm$ 741

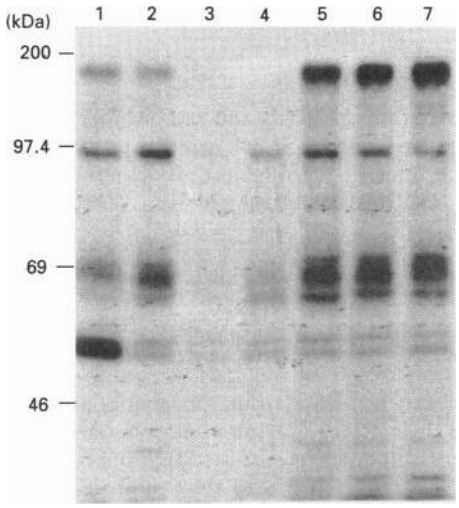

Figure 4 Effect of genistein on bombesin-stimulated protein tyrosine phosphorylation

Cells were preincubated for 1 h with vehicle or genistein, stimulated for 2 min with vehicle or a final concentration of 3 nM bombesin, and then lysed. A sample of each lysate corresponding to approx. 25 μg of protein was analysed for protein tyrosine phosphorylation as described. The numbered lanes in the photograph correspond to protein phosphotyrosine in lysates from cells treated as follows: 1, preincubated with 0.2% DMSO, stimulated with HHBG; 2, preincubated with 0.2% DMSO, stimulated with 3 nM bombesin; 3, preincubated with 100 μM genistein, stimulated with HHBG; 4, preincubated with 100 μM genistein, stimulated with 3 nM bombesin; 5, preincubated with 30 μM genistein, stimulated with 3 nM bombesin; 6, preincubated with 10 μM genistein, stimulated with 3 nM bombesin; 7, preincubated with 1 μM genistein, stimulated with 3 nM bombesin. Results are from a single experiment representative of three. The values on the left indicate the positions of molecular mass markers.

Bombesin-stimulated [^{3}H]PtdBut generation is partially due to activation of tyrosine kinases

The Western-blotting experiments demonstrated that proteins with apparent molecular masses of 110–130 kDa, 80–85 kDa and 65 kDa were phosphorylated in response to treatment of the cells with either bombesin or pervanadate. These results raised the possibility that the stimulation of PLD by bombesin could be due to the stimulation of tyrosine phosphorylation; therefore the

Table 2 Inhibition of bombesin-stimulated PLD activity by genistein

[^{3}H]Palmitate-labelled cells were preincubated for 1 h with increasing concentrations of genistein or vehicle (0.1% DMSO), before a 10 min preincubation with 30 mM butan-1-ol. Cells were then stimulated for 2 min with 3 nM bombesin. Incubations were terminated by the addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut was quantified as described in the text. Results are expressed as mean ± S.D. ($n = 3$), as a percentage of the bombesin-stimulated [^{3}H]PtdBut accumulation in the absence of genistein, and are from a single experiment typical of three. Mean basal values where vehicle or genistein alone was used in the preincubation period were subtracted before percentage values were determined. Mean basal d.p.m.: 0.1%-DMSO preincubation, 1145 ± 216; genistein only (100 μM) preincubation, 773 ± 233; 3 nM bombesin, no genistein, 2185 ± 177.

[Genistein] (μM)	Percentage of bombesin-stimulated [^{3}H]PtdBut accumulation in absence of genistein
0	100 ± 17
0.1	124 ± 29
1	104 ± 15
10	99 ± 45
30	81 ± 15
100	40 ± 10

Table 3 Effect of Ro-31-8220 and genistein on bombesin-stimulated PLD activity

[^{3}H]Palmitate-labelled cells were preincubated for 1 h with vehicle (0.1% DMSO) or 100 μM genistein, before a 10 min treatment with 10 μM Ro-31-8220 or vehicle (0.1% DMSO) and 30 mM butanol. Cells were then stimulated by addition of vehicle or 100 nM bombesin. Incubations were terminated by addition of ice-cold methanol. Chloroform extracts were prepared and the generation of [^{3}H]PtdBut was quantified as described. Results are expressed as means ± S.D. ($n = 3$), as a percentage of the bombesin-stimulated [^{3}H]PtdBut accumulation in the absence of genistein, and are from a single experiment representative of three. Mean basal values where vehicle or genistein alone was used in the preincubation period were subtracted before percentage values were determined. Mean basal d.p.m.: 0.1%-DMSO preincubation, vehicle stimulation, 525 ± 124; 0.1%-DMSO preincubation, 100 nM bombesin stimulation, 2603 ± 278; 100 μM genistein preincubation + Ro-31-8220 preincubation, vehicle stimulation, 988 ± 251.

Stimulation	PtdBut formation (% of bombesin-stimulated response)
Bombesin	100 ± 12
Bombesin + Ro-31-8220	33 ± 8
Bombesin + genistein	81 ± 10
Bombesin + Ro-31-8220 + genistein	1 ± 9

effect of a range of tyrosine kinase inhibitors was investigated. Neither erbstatin nor lavendustin significantly inhibited bombesin-stimulated protein phosphotyrosine content, nor did they affect bombesin-stimulated [^{3}H]PtdBut accumulation (results not shown). However, Figure 4 shows that 100 μM genistein consistently inhibited protein tyrosine phosphorylation in lysates from cells stimulated with 3 nM bombesin. Genistein also decreased basal protein tyrosine phosphorylation (Figure 4), and at a concentration of 100 μM significantly inhibited bombesin-stimulated PLD activity by approx. 50% ($P = 0.011 \pm 0.006$) (Table 2). The inhibitory effect of genistein was extremely variable between experiments with significant effects only observed at 100 μM.

The effect of the PKC inhibitor Ro-31-8220 and 100 μM genistein on 100 nM-bombesin-stimulated [^{3}H]PtdBut production was investigated. In the particular experiment illustrated (Table 3), 100 μM genistein had only a small effect on bombesin-stimulated [^{3}H]PtdBut generation, whereas Ro-31-8220 partially inhibited stimulated PLD activity. However, in the presence of both genistein and Ro-31-8220 the bombesin response was completely abolished.

Since at least part of the stimulated PLD activity was apparently dependent on tyrosine phosphorylation, it was considered possible that a PLD species analogous to phospholipase Cγ existed, where activity is regulated by direct tyrosine phosphorylation of the enzyme. Therefore immunoprecipitates using anti-phosphotyrosine antibodies were prepared from control and pervanadate-stimulated cells, and PLD activity was measured. No detectable PLD activity was measured in either immunoprecipitate, suggesting that a PLDγ-type is unlikely to exist. These experiments were controlled by measuring PtdIns 3-kinase activity in the immunoprecipitates, which was increased in the pervanadate-treated cells.

Regulation of PLD activity by guanine nucleotides

In addition to regulation by different kinases, it has been proposed that PLD activity is controlled through the activation of a G-protein; therefore the effects of non-hydrolysable GTP analogues

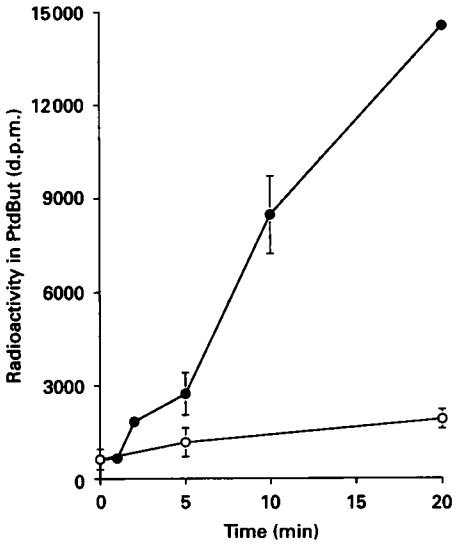

Figure 5 Time course of GTP[S]-stimulated PLD activity

[^{3}H]Palmitate-labelled cells were permeabilized with streptolysin-O, then stimulated for times indicated with 30 μM GTP[S] in the presence of 30 mM butanol. Incubations were terminated by addition of methanol, chloroform extracts were prepared and [^{3}H]PtdBut was quantified as described. Results are expressed as radioactivity in PtdBut (d.p.m.; mean ± S.D., $n = 3$) and are from a single experiment representative of three. ●, +GTP[S]; ○, control.

on PLD activity were investigated in streptolysin-O-permeabilized cells. All permeabilization experiments were performed at a free Ca^{2+} concentration of 150 nM in order to maintain physiologically relevant conditions. Despite this, the process of cell permeabilization itself was found to activate PLD, as demonstrated by the stimulation of [^{3}H]PtdBut accumulation when streptolysin-O was added to cells in the presence of 30 mM butan-1-ol (radioactivity in [^{3}H]PtdBut, mean d.p.m. ± S.D.,

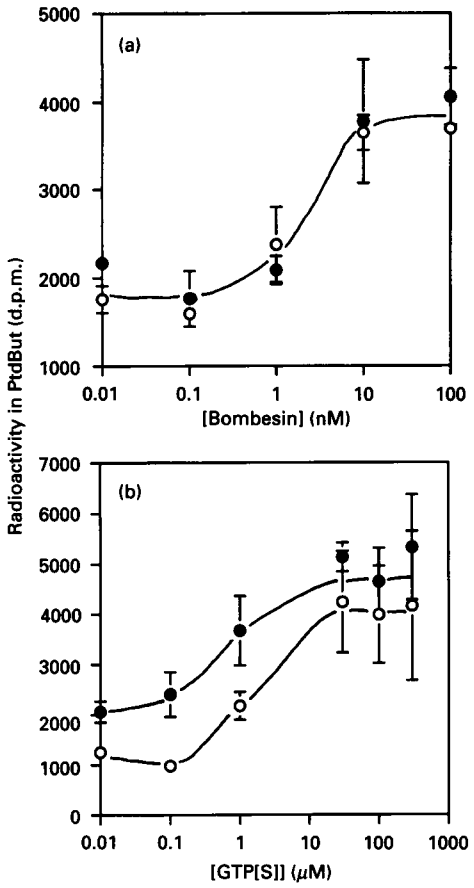

Figure 6 (a) Effect of bombesin on 30 μM-GTP[S]-stimulated PLD activity; (b) effect of GTP[S] on 3 nM-bombesin-stimulated PLD activity

(**a**) [^{3}H]Palmitate-labelled cells were permeabilized by using streptolysin-O (0.6 i.u./ml) and stimulated for 1 min with increasing concentrations of bombesin and 30 mM butanol in the absence (○) or presence (●) of 30 μM GTP[S]. Incubations were terminated by the addition of chloroform. Chloroform extracts were prepared and [^{3}H]PtdBut was quantified as described. Results are expressed as radioactivity in [^{3}H]PtdBut generated (d.p.m.; mean ± S.D., $n = 3$) and are from a single experiment typical of three. (**b**) [^{3}H]Palmitate-labelled cells were permeabilized by using streptolysin-O (0.6 i.u./ml) and stimulated for 5 min with increasing concentrations of GTP[S] and 30 mM butanol in the absence (○) or presence (●) of 3 nM bombesin. Incubations were terminated by addition of methanol. Chloroform extracts were prepared and [^{3}H]PtdBut generated was determined as described. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m.; mean ± S.D., $n = 3$) and are from a single experiment typical of three.

$n = 3$: permeabilization without butanol, 1480 ± 832; permeabilization + butanol, 4547 ± 380; data from a single experiment typical of three). Permeabilization was therefore performed in the absence of alcohol, followed by two rapid washes in buffer before stimulation in the presence of butanol.

Figure 5 shows the time course of GTP[S]-stimulated PLD activity in Swiss 3T3 cells. There was a clear lag in stimulation, but in some experiments GTP[S]-stimulated [^{3}H]PtdBut accumulation could be observed after 1 min; nevertheless stimulation was always significant after 5 min ($P = 0.015 \pm 0.015$, $n = 3$). Elevation of GTP[S]-stimulated PLD activity above basal was always significant at 1 μM guanine nucleotide concentration ($P = 0.004 \pm 0.004$, $n = 3$). The fold stimulation of [^{3}H]PtdBut accumulation in response to 5 min of GTP[S] treatment was not affected by washing cells for times up to 15 min after permeabilization (results not shown). Figure 6(a) shows that 30 μM GTP[S] did not affect bombesin-stimulated PLD activity at any concentration of bombesin tested. Figure 6(b) shows that, at low GTP[S] concentration in the presence of bombesin, there was no potentiation of stimulated [^{3}H]PtdBut generation. In some experiments a small potentiation was observed; however, in all experiments there was no potentiating effect of bombesin on concentrations of GTP[S] of 30 μM and above.

Table 4 Effect of guanine nucleotides on bombesin- and PMA-stimulated PLD activity

[^{3}H]Palmitate-labelled cells were permeabilized with streptolysin-O (0.6 i.u./ml). In (**a**) they were preincubated with 2 mM GDP[S] for 1 minute and then stimulated in the presence of 30 mM butanol with 100 nM bombesin for 5 min, or 100 nM PMA for 10 min. In (**b**) they were stimulated for 10 min with vehicle, 30 μM GTP[S], 100 nM PMA or 100 nM PMA + 30 μM GTP[S]. The generation of [^{3}H]PtdBut was determined as described in the text. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m.; mean ± S.D., $n = 3$) and are from a single experiment typical of three others in each case.

(a) Treatment	Bombesin (d.p.m. in PtdBut)	PMA (d.p.m. in PtdBut)
Control	455 ± 40	562 ± 32
Control + GDP[S]	711 ± 203	550 ± 42
Stimulated	2559 ± 427	5127 ± 839
Stimulated + GDP[S]	1664 ± 137	6725 ± 550
(b) Condition	**PtdBut (d.p.m.)**	
Control	648 ± 87	
PMA	5487 ± 250	
GTP[S]	1082 ± 82	
PMA + GTP[S]	7786 ± 500	

Table 5 Effect of kinase inhibitors on GTP[S]-stimulated PLD activity

[^{3}H]Palmitate-labelled cells were permeabilized and stimulated as described in the text. Incubations were for 20 min, and the permeabilized cells were preincubated with inhibitors for 5 min before stimulation. Concentrations used were 30 μM GTP[S], 100 nM bombesin, 10 μM Ro-31-8220, 100 μM genistein. Results are expressed as radioactivity in [^{3}H]PtdBut (d.p.m.; mean ± S.D., $n = 3$) and are from a single experiment typical of two others.

Condition	PtdBut (d.p.m.)
Control	1373 ± 524
GTP[S]	5460 ± 1068
GTP[S] + Ro-31-8220	2329 ± 254
GTP[S] + genistein	2233 ± 185
GTP[S] + Ro-31-8220 + genistein	2377 ± 496

The response to bombesin was significantly decreased by preincubation of permeabilized cells with 2 mM guanosine 5′-[β-thio]diphosphate (GDP[S]) ($P = 0.02 \pm 0.01$, $n = 3$) (Table 4). The inhibition of bombesin-stimulated [^{3}H]PtdBut generation, in the presence of GDP[S], over three separate experiments was 58 ± 19%. Phorbol 12-myristate 13-acetate (PMA)-stimulated PLD activity was unaffected by GDP[S] (Table 4), whereas 30 μM GTP[S] and 100 nM PMA were synergistic in the generation of [^{3}H]PtdBut over a 10 min stimulation period ($P = 0.01 \pm 0.01$, $n = 3$) (Table 4). Table 5 shows that the stimulation of PLD activity by GTP[S] was partly inhibited by both genistein and Ro-31-8220; however, there was no additive inhibitory effect observed.

Cells were depleted of cellular ATP by treatment with the metabolic inhibitors antimycin A and deoxyglucose, thereby preventing the activation of PLC and cellular kinases [14,25].

Table 6 Effect of cellular ATP depletion on (a) stimulated generation of total inositol phosphates and (b) stimulated PLD activity

Cells were labelled with [^{3}H]inositol and [^{3}H]palmitate, permeabilized and stimulated as described in the Materials and methods section. Cells that were to be metabolically inhibited were preincubated in buffer minus glucose containing 6 mM deoxyglucose and 5 μM antimycin A, and permeabilization, subsequent washes, preincubations and stimulations were performed in the presence of metabolic inhibitors. All cells were preincubated for 10 min after permeabilization, with 10 mM LiCl and 30 mM butanol, and then stimulated for 5 min with 30 μM GTP[S], 3 nM bombesin or 30 μM GTP[S] + 3 nM bombesin. Incubations were terminated by addition of methanol, and [^{3}H]PtdBut and total [^{3}H]inositol phosphates were quantified as described above. Results are expressed as radioactivity in (**a**) total [^{3}H]inositol phosphates and (**b**) [^{3}H]PtdBut (d.p.m.; mean $\pm$ S.D., $n = 3$) and are from a single experiment representative of three.

(**a**) [^{3}H]Inositol phosphates accumulation	Control cells	Metabolically inhibited cells
Control	842 $\pm$ 63	362 $\pm$ 60
GTP[S]	1093 $\pm$ 22	566 $\pm$ 68
Bombesin	1527 $\pm$ 127	556 $\pm$ 77
GTP[S] + bombesin	1881 $\pm$ 252	775 $\pm$ 180

(**b**) [^{3}H]PtdBut generation	Control cells	Metabolically inhibited cells
Control	1441 $\pm$ 217	663 $\pm$ 101
GTP[S]	3292 $\pm$ 206	755 $\pm$ 125
Bombesin	3153 $\pm$ 196	792 $\pm$ 14
GTP[S] + bombesin	3277 $\pm$ 453	879 $\pm$ 120

Table 6 shows that pretreatment with the metabolic inhibitors decreased the stimulation of production of total [^{3}H]inositol phosphates by 30 μM GTP[S], 100 nM bombesin or 30 μM GTP[S] + 100 nM bombesin, to near basal levels. Stimulation of PLD activity was decreased to similar levels, and, in some experiments, was completely abolished (Table 6).

DISCUSSION

The aim of this study was to define the PKC-independent mechanism of PLD activation in bombesin-stimulated Swiss 3T3 cells. We have previously demonstrated that epidermal growth factor can stimulate PLD activity in the absence of inositol lipid hydrolysis; therefore the effect of the tyrosine phosphate phosphatase inhibitor pervanadate, which clearly increased tyrosine phosphorylation (Figure 1), on phospholipase activity was examined. The stimulation of both [^{3}H]PtdBut and total [^{3}H]inositol phosphates by pervanadate was not significant above basal levels until 10 min (Figure 2), and thus lagged considerably behind that of protein tyrosine phosphorylation, which was observable within 30 s (Figure 1), suggesting that protein tyrosine phosphorylation, through inhibition of tyrosine phosphatases, could lead to activation of PtdIns-PLC and PLD activity. In most experiments the pervanadate stimulation of PLD activity in Swiss 3T3 fibroblasts occurred concomitantly with that of PtdIns-PLC activity, with the accumulation of total [^{3}H]inositol phosphates generally being lower than that of [^{3}H]PtdBut. The elevation of total [^{3}H]inositol phosphates implied that pervanadate activated PLCγ; however, it is unlikely that an equivalent PLD isoform exists, since we were unable to detect enzyme activity in immunoprecipitates prepared from pervanadate-treated cells by using anti-phosphotyrosine antibody. Nevertheless, the results support the possibility that the PKC-independent component of bombesin-stimulated PLD activity is through the activation of tyrosine kinases.

Genistein, an isoflavone and a specific competitive inhibitor with respect to ATP, was the only tyrosine kinase inhibitor, of the range tested, that consistently attenuated tyrosine phosphorylation in cells stimulated with a sub-maximal dose of bombesin (Figure 4). Concomitant with the decrease in tyrosine phosphorylation, 100 μM genistein partially decreased bombesin-stimulated PLD activity (Table 2). This concentration of genistein is below that thought to inhibit PKC activity. The failure of the erbstatin analogue and lavendustin to affect bombesin-stimulated tyrosine phosphorylation may have reflected cell-specific tyrosine kinases or poor entry into the cell.

Genistein had only a small inhibitory effect on 100 nM-bombesin-stimulated PLD activity. Though 10 μM Ro-31-8220 alone only partially attenuated 100 nM-bombesin-stimulated PLD activity, the abolition of [^{3}H]PtdBut accumulation in the additional presence of 100 μM genistein suggested that PLD activity was regulated through both PKC and tyrosine kinases (Table 3). The failure of genistein alone significantly to affect stimulated [^{3}H]PtdBut accumulation may be because the small contribution of tyrosine kinases to 100 nM-bombesin-stimulated PLD activity could not be observed within the limits of experimental error in cases where maximal doses of agonist are used.

Control of PLD by PKC-dependent and -independent pathways both imply that the activation is downstream of direct receptor–G-protein coupling. However, experiments where permeabilized cells were stimulated with GTP[S] and bombesin suggested a G-protein involvement in the regulation of PLD activity (Figure 6). The inhibition of bombesin-stimulated [^{3}H]PtdBut by GDP[S] provided further evidence for a G-protein-regulated PLD activity (Table 4); however, this inhibition was incomplete, which suggests either that a G-protein involved in regulating PLD activity exists in a GTP-bound state under resting conditions or that there is only a partial involvement of such regulatory proteins. Nevertheless, the failure of a maximal concentration of GTP[S] to affect bombesin-stimulated [^{3}H]PtdBut accumulation (Figure 6a), and the lack of potentiation of 3 nM-bombesin-stimulated [^{3}H]PtdBut accumulation by GTP[S] (Figure 6b), would suggest that the G-protein involvement was indirect.

In HL-60 cells GTP[S]-stimulated PLD activity was significantly decreased if cells were left for 15 min after permeabilization before stimulation, although the presence of GTP[S] exerted some protection against this attenuation [14]. Those authors suggested that a cytosolic factor was required for full activation of PLD, and it has been proposed that this is the low-molecular-mass G-protein ARF [19,20]. The observation that the extent of GTP[S]-stimulated [^{3}H]PtdBut accumulation was not significantly decreased, even after washing the permeabilized Swiss 3T3 cells for up to 15 min, implied that a cytosolic factor was unnecessary for stimulation of PLD activity. This was further suggested by the ability of bombesin to stimulate [^{3}H]PtdBut generation in the absence of added GTP[S]. However, it is possible that in Swiss 3T3 cells a membrane-associated form of ARF is involved in PLD activation. Alternatively, another member of the low-molecular-mass G protein family may be involved in regulating PLD in 3T3 cells, since it remains to be determined if ARF regulates PLD activity in a cell type other than HL-60. Indeed, Bowman et al. [30] have proposed that a member of the Rho family is involved in the activation of PLD in human neutrophils.

The synergy observed between PMA and GTP[S] on PLD activation, and the lack of effect of GTP[S] (Table 4), suggested

that PMA-stimulated PLD activity was not mediated through a G-protein. Both pathways converge presumably at a point subsequent to PKC activation to stimulate [^{3}H]PtdBut formation. Similar synergistic activation of PLD between PMA and non-hydrolysable GTP analogues has been reported in rabbit platelet membranes [16] and streptolysin-O-permeabilized HL60 cells [14].

An indirect G-protein/PLD coupling was further suggested by the failure of bombesin or GTP[S] to activate PLD when total cellular ATP and PtdIns-PLC activity was depleted by the presence of inhibitors of the mitochondrial respiratory chain (Table 6). This implied that a kinase intermediate was essential to the stimulation of PLD activity in Swiss 3T3 cells; an alternative explanation is that there may be a requirement for PtdInsP_2 in regulating the phospholipase, as suggested by the measurements by Brown et al. [19] *in vitro*. These findings contrasted with similar experiments in HL60 cells where, although PLC activation was minimal, significant GTP[S] stimulation of PLD activity was observed, suggestive of a direct G-protein coupling [14]. Furthermore, in rat pheochromocytoma PC12 cells ATP and Ca^{2+} were found to be essential for GTP[S]-stimulated PLD activity [31], whereas in U937 promonocytic leucocytes stimulation was found to be regulated by both ATP-dependent and -independent pathways [32]. In order to assess the involvement of a kinase pathway in the GTP-dependent stimulation of PLD, inhibitor effects on GTP[S]-stimulated activity were examined (Table 5). The partial non-additive effects of the PKC and tyrosine kinase inhibitors suggested that the G-protein-regulated pathway is partly kinase-dependent. The results, however, do not permit a determination of whether the kinases function upstream or downstream of the putative G-protein, since the use of inhibitors in permeabilized cells may have non-selective effects. However, bombesin may activate tyrosine kinases independently of GTP-binding proteins. Depletion of cellular ATP would have abolished any kinase activity, in addition to PLC-catalysed PtdIns(4,5)P_2 hydrolysis. A direct stimulation of PLD through kinases would therefore not have been distinguished from that regulated by G-proteins.

In conclusion, the regulation of bombesin-stimulated PLD activity in Swiss 3T3 cells appears to involve both protein kinase C and tyrosine kinase activation. The relative importance of the two kinases in the control of PLD activity may differ between cell types and/or agonist. The mechanism of PLD activation by protein tyrosine phosphorylation is unclear, especially without knowledge of the kinases involved and their possible substrates. Possible pathways of regulation include direct phosphorylation of PLD on tyrosine residues, or removal of a constitutive inhibitor upon protein tyrosine phosphorylation. Alternatively, proteins regulating the function of ARF, or an alternative low-molecular-mass G-protein, may be substrates for both tyrosine kinases and PKC. It is also possible that the bombesin-stimulated activation of G-protein- and tyrosine kinase-mediated second-messenger pathways may act together or in parallel to stimulate the same or different PLD isoforms. Clarification of these possibilities awaits the purification and cloning of the PLD enzyme(s).

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