Diacylglycerol generated by exogenous phospholipase C activates the mitogen-activated protein kinase pathway independent of Ras- and phorbol ester-sensitive protein kinase C: dependence on protein kinase C-ζ

Marc C. M. van DIJK, Francisco J. G. MURIANA*, Paul C. J. van der HOEVEN, John de WIDT, Dick SCHAAP†, Wouter H. MOOLENAAR and Wim J. van BLITTERSWIJK‡

Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

The role of diacylglycerol (DG) formation from phosphatidylcholine in mitogenic signal transduction is poorly understood. We have generated this lipid at the plasma membrane by treating Rat-1 fibroblasts with bacterial phosphatidylcholine-specific phospholipase C (PC-PLC). This treatment leads to activation of mitogen-activated protein kinase (MAPK). However, unlike platelet-derived growth factor (PDGF) or epidermal growth factor (EGF), PC-PLC fails to activate Ras and to induce DNA synthesis, and activates MAPK only transiently (< 45 min). Down-regulation of protein kinase C (PKC) -α, -δ and -ε isotypes has little or no effect on MAPK activation by either PC-PLC or growth factors. However, Ro 31-8220, a highly selective inhibitor of all PKC isotypes, including atypical PKC-ζ but not Raf-1, blocks MAPK activation by PDGF and PC-PLC, but not that by EGF, suggesting that atypical PKC mediates the PDGF and PC-PLC signal. In line with this, PKC-ζ is activated by PC-PLC and PDGF, but not by EGF, as shown by a kinase assay *in vitro*, using biotinylated ε-peptide as a substrate. Furthermore, dominant-negative PKC-ζ inhibits, while (wild-type) PKC-ζ overexpression enhances MAPK activation by PDGF and PC-PLC. The results suggest that DG generated by PC-PLC can activate the MAPK pathway independent of Ras and phorbol-ester-sensitive PKC but, instead, via PKC-ζ.

INTRODUCTION

Growth factors transduce their signals from receptors at the cell surface to the nucleus via activation of the small GTP-binding protein $p21^{ras}$ (Ras) and a cascade of protein kinases, in which Raf-1, mitogen-activated protein kinase kinase (or MEK) and mitogen-activated protein kinase (MAPK or ERK) are sequentially activated [1]. Raf-1 is recruited to the plasma membrane by Ras-GTP and is subsequently activated by an interplay of other kinases [2,3], scaffold proteins [4,5] and lipids [6] by mechanisms which are poorly understood [7].

Most mitogens, including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), elicit phospholipid breakdown, not only polyphosphoinositide hydrolysis, but also phosphatidylcholine (PC) breakdown by PC-specific phospholipases C (PC-PLC) and D (for reviews, see [8,9]). Whether PC hydrolysis plays a role in receptor-mediated MAPK activation and subsequent nuclear events is not clear. Since PC hydrolysis leads to diacylglycerol (DG) formation without mobilizing Ca^{2+}, it has been suggested to represent an alternative route for protein kinase C (PKC) stimulation, particularly the 'new' Ca^{2+}-independent isotypes such as PKC-ε and -δ, for prolonged time periods [10]. While, in some systems, conventional PKC isotypes can phosphorylate and activate Raf-1 [2,11], the possibility remains that DG may be capable of activating the MAPK pathway via other (PKC?) target enzymes.

In the present study, we have treated Rat-1 fibroblasts with *Bacillus cereus* PC-PLC to raise endogenous levels of DG in an attempt to mimic receptor-mediated PC hydrolysis. Such PC-PLC treatment appeared to be mitogenic for Swiss 3T3 fibroblasts [12] and a macrophage cell line [13] by a PKC-independent unknown mechanism. We report here, however, that in Rat-1 fibroblasts, *B. cereus* PC-PLC is not mitogenic, yet is capable of activating MAPK transiently, independently of Ras and phorbol-ester-sensitive PKC. Instead, we present data suggesting that the atypical PKC-ζ is an essential mediator of MAPK activation in PC-PLC- and PDGF-stimulated cells.

EXPERIMENTAL

Materials and cell culture

[^{3}H]Myristic acid was obtained from Du Pont–New England Nuclear. [^{32}P]Orthophosphate, [γ-^{32}P]ATP, [^{3}H]thymidine and the enhanced chemoluminescence system were obtained from Amersham. EGF was from Collaborative Research. Human recombinant PDGF-B/B, PC-PLC (from *B. cereus*), sphingomyelinase (from *B. cereus*), 8-Br-cAMP, dithiothreitol, leupeptin and BSA (fraction V) were from Boehringer Mannheim. Trypsin inhibitor (type II-O), PMSF, myelin basic protein and lipid standards were obtained from Sigma. 1,2-Dioctanoylglycerol (diC8) was from Avanti Polar Lipids Inc., C-6 ceramide from Biomol, PMA and phorbol 12,13-dibutyrate (PDBu) were

Abbreviations used: DMEM, Dulbecco's modified Eagle's Medium; EGF, epidermal growth factor; DG, diacylglycerol; diC8, 1,2-dioctanoylglycerol; GST, glutathione *S*-transferase; MAPK (ERK), mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PKC, protein kinase C; PDGF, platelet-derived growth factor; PA, phosphatidic acid; PDBu, phorbol 12,13-dibutyrate.

* Present address: Instituto de la Grasa (CSIC), Apartado 1078, 41012 Sevilla, Spain.

† Present address: Intervet International, Department of Parasitology, P.O. Box 31, 5830 AA Boxmeer, The Netherlands.

‡ To whom correspondence should be addressed.

from LC Services Corporation, Woburn, MA, U.S.A.; aprotinin was from Fluka and orthovanadate from BDH. Ro 31-8220 (compound 3, [14]) was kindly provided by the Roche Research Centre (Welwyn Garden City, Herts, U.K.). All further chemicals were of analytical grade. Protein G–Sepharose 4 Fast Flow and Protein A–Sepharose CL-4B were from Pharmacia. Dulbecco's modified Eagle's Medium (DMEM) was obtained from Gibco and minimal essential medium (without phosphate) from Flow. Rat-1, Swiss 3T3 and COS7-M6 cells were routinely grown in 5-cm plastic dishes in DMEM containing 7.5% fetal calf serum (FCS; Life Technologies Inc.) to 80% confluency. Cells were serum-starved for 24 h prior to stimulation.

cDNA constructs and antibodies

pEXV-ERK2-tag contains murine ERK2 with a C-terminal epitope-tag from c-*myc* [15]. pmtSM-PKC-ζ and pmtM-Raf-1 have been described previously [15,16]; pmtM and pmtSM are pMT2-derived expression vectors [15]. Mouse PKC-ζ-K281W (kinase-inactive) in a pcDNAI-Amp vector [17] was kindly provided by Dr S. Gutkind (NIH, Bethesda, MD, U.S.A.), and was subcloned in the pmtSM vector for our transfection experiments.

Monoclonal anti-Ras (Y13-259) was from Oncogene Science, monoclonal anti-Raf-1 (R19120) was obtained from Transduction Laboratories (Lexington, KY, U.S.A.), polyclonal anti-PKC-ι (N-terminal epitope) was from Santa Cruz Biotechnology. Polyclonal anti-PKC-α, -β, -γ and -ϵ were kindly provided by P. Parker (Imperial Cancer Research Fund Laboratories, London, U.K.). Polyclonal anti-PKC-δ was from Gibco. Polyclonal anti-PKC-ζ and anti-MAPK (ERK2) were raised against C-terminal peptides of these proteins. Polyclonal anti-PKC-λ was kindly provided by Dr S. Ohno (Yokohama City University School of Medicine, Yokohama, Japan). Monoclonal antibody 9E10 is directed against a C-terminal epitope of c-*myc*. Peroxidase-conjugated antibodies, normal mouse serum and rabbit-anti-mouse serum were from DAKO (Gastrup, Denmark).

Determination of lipid metabolites

Cells were labelled with [^{3}H]myristic acid and extracted as described previously [18]. The radiolabel was incorporated mainly (85%) in to the PC fraction of the phospholipids. Lipids were separated by one-dimensional TLC (Silica-gel 60; Merck), in four runs and the plates were dried after each run. The first two runs were in hexane/diethyl ether/methanol (4:1:1; by vol.) for the full length of the plate. The plate was then sprayed with primuline to allow identification of the lipid standards. The third and fourth runs were with the upper phase of ethyl acetate/iso-octane/acetic acid/water (13:2:3:10; by vol.) up to the position of the non-esterified fatty acids and monoacylglycerol respectively. This composite TLC system allowed separation of triacylglycerol, 1,3-DG, 1,2-DG, non-esterified fatty acids, monoacylglycerol, phosphatidic acid (PA) and the remainder of the phospholipids, including PC (in decreasing order of R_f). Spots were scraped off and the radioactivity was determined by liquid scintillation spectroscopy.

DNA synthesis

Serum-starved cells in 24-well plates were incubated for 20 h with 0.5 μCi [^{3}H]thymidine in the presence or absence of agonist. Cells were washed with PBS and methanol-precipitated material was dissolved in 0.1 N NaOH. The radioactivity incorporated was quantified by liquid scintillation spectroscopy.

Determination of GTP and GDP bound to Ras

Cells in 6-well plates were labelled for 16 h in phosphate-free medium with 250 μCi [^{32}P]orthophosphate per well, and stimulated for 10 min. Cells were lysed in lysis buffer [50 mM Hepes, pH 7.4/150 mM NaCl/5 mM $MgCl_2$/1 mM K_2HPO_4/10 mM NaH_2PO_4/1 mM PMSF/0.2 mM Na_3VO_4/1 mM NaF/1 mM ATP/0.1 mM GDP/0.1 mM GTP containing 1% (v/v) Triton X-114, 1% (w/v) BSA, 1 μg/ml of aprotinin, 10 μg/ml of trypsin inhibitor and 1 μg/ml of leupeptin]. Ras was immunoprecipitated with monoclonal antibody Y13-259 and Protein G–Sepharose. Guanine nucleotides were eluted, separated on PEI–cellulose F TLC plates (Merck) and autoradiographed [19]. The [^{32}P]-GTP/(GDP + GTP) ratio was determined by liquid scintillation spectroscopy.

MAPK/ERK2 phosphorylation and activity

Stimulated cells were washed with cold PBS and lysed in SDS sample buffer. Proteins were separated by SDS/PAGE (10% acrylamide, 0.4% bisacrylamide gel) and transferred to nitrocellulose membranes. These were blocked for 30 min with 5% milk powder in TBST [10 mM Tris/HCl, pH 8.0/150 mM NaCl containing 0.05% (v/v) Tween-20], incubated with anti-p42 MAPK (ERK2) antibodies, washed with TBST and subsequently incubated with peroxidase-conjugated second antibody. Immunostained antibodies were visualized by enhanced chemiluminescence. A reduced electrophoretic mobility (gel-shift) is indicative for MAPK phosphorylation and activation. To analyse kinase activity of MAPK, cells were transfected ($CaCl_2$-phosphate protocol) with pEXV-ERK2 tagged with a C-terminal epitope from c-*myc*, with or without pmtSM-PKC-ζ, as described [15], and stimulated 2 days later after 24-h serum starvation. The cells were lysed in 50 mM Tris/HCl, pH 7.5/100 mM NaCl/5 mM EDTA/10 mM NaF/40 mM β-glycerophosphate/0.2 mM Na_3VO_4/0.1 mM PMSF containing 1% (v/v) Triton X-100, 1 μg/ml of aprotinin and 1 μg/ml of leupeptin, and subsequently immunoprecipitated with 9E10 monoclonal antibody directed against the tag. Kinase activity of the precipitated ERK2 was determined *in vitro* using myelin basic protein (250 μg/ml) as a substrate in 20 mM Hepes/10 mM $MgCl_2$/1 mM dithiothreitol/10 mM *p*-nitrophenol phosphate/50 μM ATP and 5 μCi [γ-^{32}P]ATP per sample. After shaking for 15 min at 30 °C, the reaction was stopped by the addition of 10 μl of formic acid. Supernatant (40 μl) was spotted onto P81 phosphocellulose paper (Whatman). The paper was dried, washed extensively in 0.15 M phosphoric acid and counted by liquid scintillation spectroscopy. Alternatively, the reaction was terminated by the addition of SDS sample buffer and analysed by SDS/PAGE (15% gels). The radioactivity incorporated into myelin basic protein was quantified using a PhosphorImager.

PKC-ζ assay in immunoprecipitates

The best substrate *in vitro* for PKC-ζ known to date is ϵ-peptide (ERMRPRKRQGSVRRRV), derived from the pseudosubstrate region of PKC-ϵ [20,21]. We coupled ϵ-peptide covalently to biotin and used this as a substrate in a kinase assay *in vitro* with [γ-^{32}P]ATP. Subsequent binding to streptavidin beads facilitates isolation (filtration and washing) and quantification of incorporated ^{32}P, with very low standard errors (P. C. J. van der Hoeven and W. J. van Blitterswijk, unpublished work). Cells, in 8.5-cm dishes, were stimulated and subsequently lysed with non-denaturating lysis buffer (LB) [10 mM Tris/HCl,

pH 7.4/150 mM NaCl/1 mM EDTA/1 mM EGTA/0.2 mM Na_3VO_4/0.2 mM PMSF containing 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 μg/ml of aprotinin and 1 μg/ml of leupeptin]. Lysates were clarified by centrifugation (15000 ***g*** for 10 min) and precleared twice with 3 μl of normal rabbit serum and Protein A–Sepharose for 1 h. PKC-ζ was immunoprecipitated with 3 μl of polyclonal antibodies and 10 μl of Protein A–Sepharose. Preclears and immunoprecipitates were washed with LB and with kinase buffer (20 mM Hepes, pH 7.5/10 mM $MgCl_2$/0.2 mM EGTA/0.5 mM Na_3VO_4 containing protease inhibitors) and subjected to the kinase assay *in vitro* in the same buffer containing [γ-^{32}P]ATP (1.5×10^6 c.p.m.; 600 c.p.m./pmol) and biotinylated ε-peptide (50 μM). To assess the specificity for PKC-ζ, assays were done in the presence or absence of 40 μM pseudo-ζ peptide (SIYRRGARRWRKL) or irrelevant control peptide (IETVDNKASTRAY), both preincubated for 5 min. The non-specific binding of radioactivity to the GF/A filters (Whatman) was determined in the presence of a 500-fold excess of biotin (20 mM) and did not exceed 8% of the [^{32}P]-ε-peptide control values. This value was subtracted from the results obtained.

Kinase assays in COS cell lysates

COS7 cells were transfected with pmtSM-PKC-ζ or with pmtM-Raf-1. Activation of overexpressed Raf-1 was achieved by stimulation of the cells with EGF (50 ng/ml). Raf-1 activity was determined *in vitro* by autophosphorylation and phosphorylation of its natural substrate, MEK1. The latter was used as a glutathione *S*-transferase (GST)–MEK1 fusion protein, made from a hamster MEK1 cDNA (kindly provided by J. Pouysségur, Nice, France). Cells were lysed in LB (see above), precleared and Raf-1 was immunopreciptated with monoclonal antibody. Immunoprecipitates were washed three times in LB, twice in 10 mM Pipes, pH 7.0/100 mM NaCl, and once in kinase buffer (20 mM Pipes, pH 7.0/10 mM $MnCl_2$ containing 1 μg/ml of aprotinin). Immunoprecipitates were incubated in 50 μl of this buffer with or without 1 μg of GST–MEK1 and 10 μCi [γ-^{32}P]ATP for 30 min at 30 °C with mild shaking. Overexpressed PKC-ζ was assayed in cell lysates with [γ-^{32}P]ATP in the presence or absence of PA (100 μM) as described in detail previously [16]. The reactions were terminated by the addition of SDS sample buffer and analysed by SDS/PAGE (10% gels) and autoradiography.

RESULTS

Exogenous PC-PLC generates DG in Rat-1 fibroblasts: lack of a mitogenic response

We measured DG formation in Rat-1 fibroblasts upon treatment of cells with *B. cereus* PC-PLC at an optimally effective dose of 1.0 unit/ml (doses > 2 units/ml were often toxic). Figure 1 shows that DG rapidly increases some 6-fold and, after 30 min, remains 4-fold elevated for at least 6 h. There is no sign of conversion of DG to PA.

Table 1 shows that PC-PLC fails to induce DNA synthesis, contrary to reports in other cell systems [12,13], while the growth factors EGF and PDGF are perfectly capable of doing so. The phorbol ester PMA, which is a strong activator of PKC, also is not mitogenic for Rat-1 cells. PC-PLC and PMA, in contrast to EGF and PDGF, also fail to induce GTP loading of Ras (Table 1), which is in line with the important function of Ras activation in mitogenic signalling.

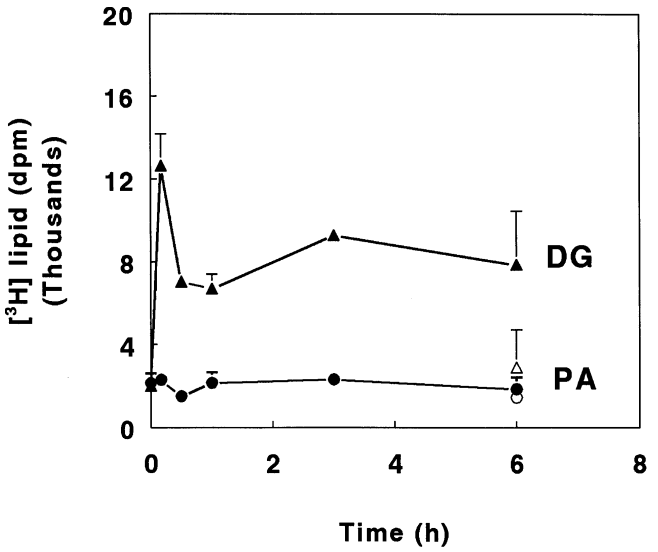

Figure 1 PC hydrolysis by treatment of cells with *B. cereus* PC-PLC

Rat-1 cells were labelled for 3 h with [^{3}H]myristic acid (2 μCi/dish) and treated for the indicated time periods with DMEM (control, open symbols) or PC-PLC (1.0 unit/ml) (filled symbols). Lipids were extracted and the radioactivity in DG (▲) and PA (●) was determined (the values are the means ± S.D.).

Table 1 Induction of DNA synthesis and activation of Ras

Rat-1 cells were stimulated for 20 h in the presence of 0.5 μCi [^{3}H]thymidine. Relative incorporation of ^{3}H into DNA is given as the means ± S.D of quadruplicates. Ras activation was determined as described in the Experimental section. After metabolic labelling with [^{32}P]orthophosphate, cells were stimulated for 10 min, Ras was immunoprecipitated and binding to [^{32}P]guanine nucleotides was estimated. The relative amounts of [^{32}P]GTP bound to Ras is given as the means ± S. E. of three experiments carried out in duplicate.

Stimulus	[^{3}H]Thymidine incorporation (fold increase)	Ras-GTP accumulation (fold increase)
Medium control	1.0 ± 0.06	1.00 ± 0.10
EGF (50 ng/ml)	22.8 ± 0.9	3.75 ± 0.20
PDGF (25 ng/ml)	14.9 ± 0.9	2.15 ± 0.11
PC-PLC (1.0 unit/ml)	1.3 ± 0.1	1.15 ± 0.08
PMA (100 nM)	1.1 ± 0.2	1.05 ± 0.08

MAPK activation by DG: no involvement of classical and 'new' PKCs

We examined activation of MAPK (p42 and p44, ERK2 and ERK1 respectively) by PC-PLC and PDGF, as estimated by the electrophoretic mobility-shift assay, which tightly associates with activation [22]. Figure 2, upper panel shows that PC-PLC stimulates MAPK activity in a dose-dependent fashion. Maximal activation is achieved at 1–2 units/ml. Other methods of generating or introducing DG into the plasma membrane also lead to MAPK activation; for instance, short-chain, membrane-permeable diC8 (25–100 μM) (Figure 2, upper panel) and phosphatidylinositol-PLC [23] (results not shown) activate MAPK, albeit generally to a lower extent than does PC-PLC. In contrast,

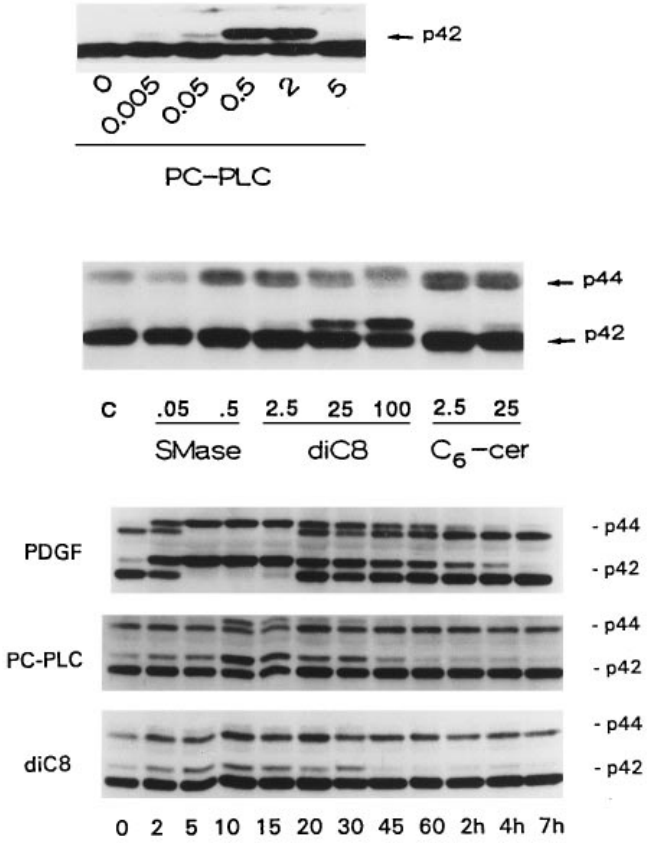

Figure 2 Dose- and time-dependent phosphorylation of MAPK by bacterial phospholipases and their lipid products, compared with PDGF and EGF

Rat-1 cells were stimulated for 10 min with the indicated concentrations of *B. cereus* PC-PLC (units/ml), *B. cereus* sphingomyelinase (SMase) (units/ml), diC8 (μM) or C-6 ceramide (μM) (upper panel). Cells were stimulated for different time periods (min up to 60 min or h) with PDGF (25 ng/ml), *B. cereus* PC-PLC (1.0 unit/ml) or diC8 (100 μM) (lower panel). Total cell lysates were immunoblotted with anti-p42 MAPK antibodies, which recognize the p42 and p44 isoforms of MAPK (indicated on the right of the Figure). The phosphorylated forms of MAPK show reduced mobility when separated by SDS/PAGE.

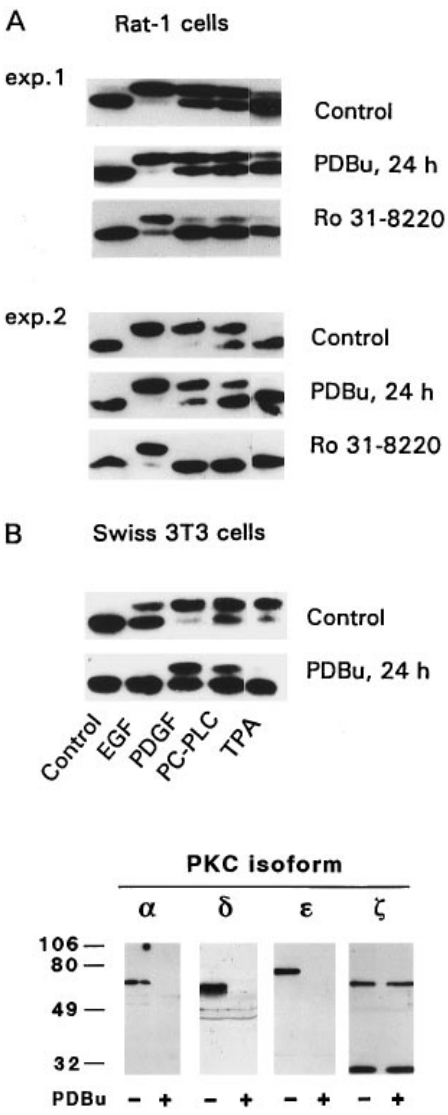

Figure 3 Effects of down-regulation or inhibition of PKC on phosphorylation of MAPK by various agents

The electrophoretic mobility shift of the p42 isoform of MAPK (ERK2) is shown here. Rat-1 (**A**) or Swiss 3T3 cells (**B**) were pretreated with either 200 nM PDBu for 24 h or with 10 μM Ro 31-8220 for 10 min, or with DMSO vehicle (Control). Cells were stimulated for 10 min with EGF (50 ng/ml), PDGF (25 ng/ml), *B. cereus* PC-PLC (1.0 unit/ml) or PMA (TPA) (100 nM). Total cell lysates were separated by SDS/PAGE and immunoblotted with anti-MAPK antibodies. For Rat-1 cells, two representative experiments (**A**, exp. 1 and exp. 2) of four giving similar results are shown. The effect of PDBu on the expression of PKC isotypes in Rat-1 cells is shown in the immunoblots (bottom panel).

ceramide generation at, or insertion into, the plasma membrane (results not shown) by treatment of cells with sphingomyelinase or the membrane-permeable C-6 ceramide respectively, do not activate MAPK in Rat-1 cells (Figure 2, upper panel). This argues against a possible non-specific membrane perturbing effect of such treatments of cells.

Figure 2 (lower panel) shows that MAPK activation by PC-PLC and diC8 is transient, being maximal at 10 min, and gradually decreasing to background within 45 min, whereas that induced by PDGF (or EGF, results not shown) is prolonged (> 4 h). This result is consistent with the notion that sustained MAPK activation correlates with mitogenic potency [24].

An obvious question is whether DG activation of MAPK would be mediated by 'classical' cPKC or 'new' nPKC [10]. PKC-α, -δ and -ϵ, the isotypes found to be expressed in Rat-1 cells, were down-regulated completely by prolonged treatment of cells with the phorbol ester, PDBu (see immunoblots, Figure 3, bottom panel). Unexpectedly, however, such PKC down-regulation has little or no effect on MAPK activation by any of the stimuli, not even by PC-PLC (Figure 3A). Conversely, treatment with PMA (for 10 min), which activates PKC, has little effect on MAPK (see also [25] and Figure 5). In contrast, under the same conditions, PMA does induce MAPK activation in Swiss 3T3 cells, while long-term pretreatment of these cells with PDBu blocks this activation (Figure 3B). These results indicate that MAPK activation by PMA (via PKC) is cell-type specific and that, in Rat-1 cells, phorbol-ester-responsive PKC isotypes fail to couple to the MAPK pathway. Despite the fact that, in Swiss 3T3 cells, PKC activation results in MAPK activation, down-regulation of PKC in these cells only partially abolishes MAPK activation induced by PC-PLC and PDGF.

Table 2 PKC-ζ activation by PDGF and PC-PLC but not by EGF; inhibition by Ro 31-8220 and by the pseudosubstrate peptide of PKC-ζ

Cells were stimulated for 10 min and cell lysates were prepared. Preclears and PKC-ζ immunoprecipitates were subjected to a kinase assay *in vitro* described in the Experimental section, with biotinylated ε-peptide as a substrate in the presence or absence of 40 μM pseudosubstrate, or control peptide or Ro 31-8220 inhibitor. The values are the [^{32}P]-ε-peptide averages obtained from 7 independent experiments done in triplicate or quadruplicate (mean error less than 6%) and normalized to equal constitutive PKC-ζ activity (control; unstimulated cells, ranging from 4–10 × 10^3 c.p.m. per experiment). Significance, determined by paired Student's *t* test; *, $P < 0.01$; **, $P < 0.0001$.

	PKC-ζ-pseudosubstrate peptide addition (percentage stimulation of PKC-ζ activity)	
	−	+
Control	100.0 ± 6.2	5.7 ± 2.2
+ Control peptide	99.5 ± 4.0	
+ Ro 31-8220 (5 μM)	8.7 ± 2.0	
EGF (50 ng/ml)	104.8 ± 7.2	6.2 ± 1.2
PDGF (25 ng/ml)	123.6 ± 4.0*	7.8 ± 2.0
PC-PLC (1.0 unit/ml)	136.8 ± 4.4**	2.1 ± 1.4
Preclear	6.5 ± 2.3	

We conclude that DG generated by PC-PLC can activate the MAPK pathway independently of DG- and phorbol-ester-responsive PKC isotypes.

Involvement of PKC-ζ

While phorbol ester is ineffective, treatment of Rat-1 cells with Ro 31-8220 (10 μM), a highly selective inhibitor of all PKC isotypes [14,26,27] including atypical PKC-ζ [16], almost completely inhibits MAPK activation by PDGF and PC-PLC. In contrast, MAPK activation by EGF is virtually unaffected (Figure 3A). These results suggest the possible involvement of atypical PKC in MAPK activation by PC-PLC and PDGF. Using specific antibodies against the atypical isoforms PKC-ζ, -λ and -ι, we found that only PKC-ζ is expressed in Rat-1 cells, and is not down-regulated by prolonged PDBu treatment (Figure 3, bottom panel).

To further test the involvement of PKC-ζ, we developed a kinase assay *in vitro* for PKC-ζ using biotinylated ε-peptide as a substrate (see the Experimental section). Using this sensitive assay, we found that PC-PLC and PDGF stimulate PKC-ζ activity up to approx. 1.4 fold, whereas EGF has no effect (Table 2). That the kinase activity indeed represents PKC-ζ is demonstrated by its complete inhibition in the presence of a PKC-ζ pseudosubstrate peptide but not with an irrelevant control peptide (Table 2). Ro 31-8220 (5 μM) also completely abrogates PKC-ζ activity (Table 2). We eliminated the possibility that Ro 31-8220 might affect autophosphorylation of the PDGF receptor (results not shown) or other kinases in the MAPK pathway, notably Raf-1 and MEK1. These kinases are apparently not affected by Ro 31-8220 in the MAPK cascade activated by EGF (Figure 3A). To confirm this, we activated Raf-1 in transfected COS cells in response to EGF, as described previously [15] and showed that the kinase activity *in vitro* of Raf-1 towards GST–MEK1 is not inhibited by treating either the cells or the Raf-1 immunoprecipitate with Ro 31-8220 (Figure 4). Ro 31-8220 also did not affect kinase activity of GST–MEK1 *in vitro*, but clearly inhibited PKC-ζ expressed in COS cells (Figure 4) [16].

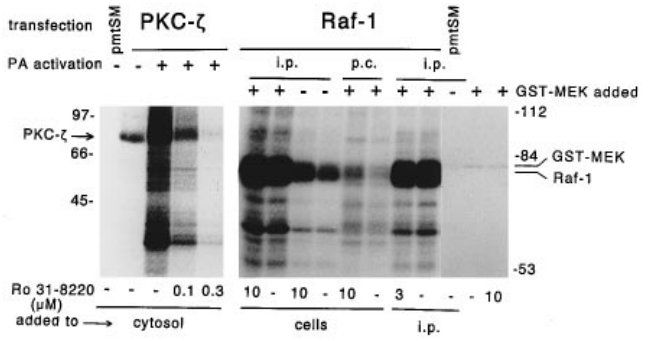

Figure 4 Ro 31-8220 inhibits PKC-ζ but not Raf-1 overexpressed in COS cells, or MEK1

COS7-M6 cells were transfected with PKC-ζ, Raf-1 or empty vector (pmtSM) where indicated. (Left panel) Cytosol from PKC-ζ or empty vector transfected COS cells were subjected to a kinase assay *in vitro*, as described previously [16], in the presence (+) or absence (−) of PA (100 μM) as activator of PKC-ζ, and Ro 31-8220 (at the concentrations shown). (Right panel) Raf-1 or pmtSM transfected COS cells were stimulated with 50 ng/ml EGF for 10 min, lysed in non-denaturating lysis buffer and Raf-1 immunoprecipitations (i.p.) and preclears (p.c.) were subjected to a kinase assay *in vitro* in the presence (+) or absence (−) of 1 μg of GST–MEK1 per assay (see Experimental section). Ro 31-8220 (at the concentrations indicated) was either preincubated for 10 min with the cells, or added to the immunoprecipitates (i.p.) in the kinase assay. Control samples were treated with DMSO vehicle. Assay mixtures were separated by SDS/PAGE and subjected to autoradiography. As a control, the results of autophosphorylation of GST–MEK1 (1 μg) alone, or with or without 10 μM Ro 31-8220 are shown in the two lanes on the extreme right. The positions of (auto)phosphorylated PKC-ζ (arrow), Raf-1 or GST–MEK1, and molecular weight markers (kDa) are indicated.

To prove that PKC-ζ mediates MAPK activation by PDGF and PC-PLC, we transiently transfected Rat-1 cells with c-myc-epitope-tagged MAPK (ERK2) together with either (wild-type) PKC-ζ or dominant-negative PKC-ζ (kinase-dead PKC-ζ-K281W) or the empty vector. Tagged MAPK was immunoprecipitated from stimulated cells and its activation was analysed by phosphorylation of myelin basic protein. Figure 5 shows that overexpression of PKC-ζ enhances, while dominant-negative PKC-ζ inhibits MAPK activation by PDGF and PC-PLC. EGF-activated MAPK remains virtually unaffected, whereas the transfections do not alter basal MAPK activity in unstimulated or PMA-treated cells.

Taken together, our results demonstrate that PDGF and PC-PLC activate PKC-ζ in Rat-1 cells, and that PKC-ζ mediates activation of the MAPK pathway by these stimuli. This signalling process is blocked by Ro 31-8220, through inhibition of PKC-ζ but not of Raf-1, MEK1, MAPK and the PDGF receptor. These results, therefore, indicate that activation of the MAPK pathway by PDGF and PC-PLC requires PKC-ζ activation, whereas PKC-ζ is not involved in EGF-induced MAPK activation.

DISCUSSION

In this paper we have studied the role of receptor-activated PC hydrolysis and consequent DG formation in mitogenic signalling. In a simplified approach, we used exogenous (bacterial) PC-PLC or a short-chain DG analogue (diC8) to generate or insert DG into the plasma membrane directly, circumventing receptor stimulation that would also generate other, possibly interfering, signals.

A major outcome of this study is that DG, besides activation of cPKC and nPKC [10], can trigger activation of the MAPK pathway in Rat-1 fibroblasts independently of these PKC iso-

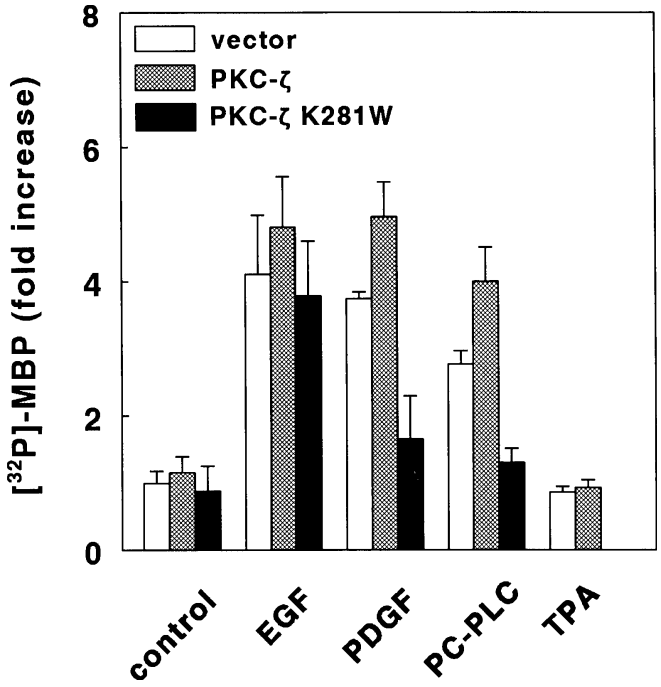

Figure 5 Dominant-negative PKC-ζ inhibits, while overexpressed wild-type PKC-ζ enhances MAPK activation by PDGF and PC-PLC, but not by EGF

Rat-1 cells were transfected with wild-type PKC-ζ (hatched bars), dominant-negative PKC-ζ-K281W (solid bars) or empty pmtSM vector (open bars), together with myc-epitope-tagged ERK2. Cells were stimulated for 10 min with EGF (50 ng/ml), PDGF (25 ng/ml), PC-PLC (1.0 units/ml), PMA (TPA) (100 nM) or DMEM (control). The cells were lysed and ERK2 immunoprecipitates were subjected to a kinase assay *in vitro* with myelin basic protein (MBP) as a substrate (see Experimental section). The values are the means (±S.D.) of three experiments in duplicate, expressed as fold stimulation relative to (vector-transfected) control cells. Quantification of radioactivity was by PhosphorImager.

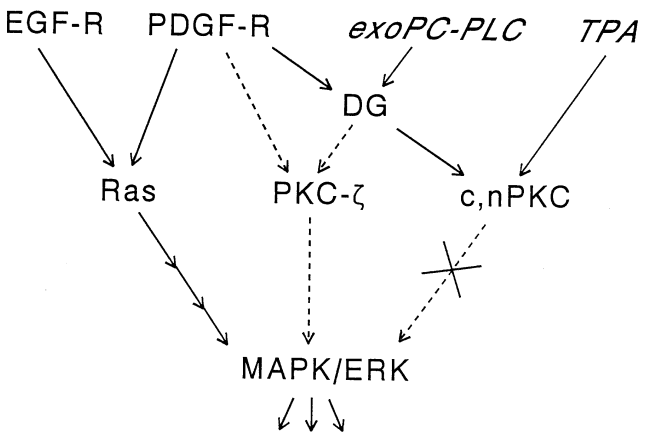

Figure 6 Summarizing scheme of signalling pathways activated in Rat-1 fibroblasts by stimuli used in this study

Although DG may activate classical/new PKC isotypes, as does phorbol ester (PMA or TPA), MAPK (ERK) activation is for the greater part independent of these phorbol ester-/DG-sensitive PKCs. Exogenous PC-PLC (from *B. cereus*) (exoPC-PLC) and the PDGF receptor activate PKC-ζ, selectively inhibitable by Ro 31-8220 and being essential for MAPK activation by these stimuli. In contrast with growth factors, exoPC-PLC treatment does not activate Ras. Dashed arrows denote novel but mechanistically undefined signalling pathways.

types. Instead, our results unexpectedly point to a critical involvement of the atypical PKC-ζ. In addition, the MAPK cascade is activated by PC-PLC (DG) in a manner that bypasses Ras activation (Figure 6). We have demonstrated that the complete down-regulation of all phorbol ester-responsive PKC isotypes (PKC-α, -δ and -ε) expressed in Rat-1 cells has little or no effect on PC-PLC- or growth factor-activated MAPK. The highly selective PKC inhibitor Ro 31-8220, however, which also strongly inhibits PKC-ζ (Table 2 and Figure 4) [16], was found to abrogate MAPK activation by PDGF and PC-PLC but not by EGF. Ro 31-8220 (10 μM) did not inhibit other protein kinases in the signalling pathway, including the PDGF receptor, Raf-1, MEK1 or MAPK. We were able to measure PKC-ζ activation in cells, using a novel assay with biotinylated ε-peptide as a substrate. PKC-ζ is constitutively active in resting cells (as also shown by others, see [28]), while its overall activity is enhanced by PC-PLC and PDGF, but not by EGF. Although this activation does not exceed 40 % of the control levels, it is highly reproducible and statistically significant, thanks to the sensitivity of the assay. Conceivably, this moderate increase in overall activity might represent only a fraction of total PKC-ζ, i.e. that localized in the particular signalling compartment of the cell. Furthermore we found that MAPK activation by PDGF and PC-PLC is inhibited by dominant-negative PKC-ζ and is enhanced upon overexpression of (wild-type) PKC-ζ in transfected Rat-1 cells. These transfections did not affect MAPK activation induced by EGF, nor the basal MAPK activity in control or PMA-treated cells. Together, these results indicate that PDGF and PC-PLC (DG) but not PMA activate the MAPK pathway in a PKC-ζ-dependent fashion. By contrast, MAPK activation by EGF is independent of PKC-ζ.

PC-PLC activates MAPK only transiently and is not mitogenic for Rat-1 cells, in agreement with results in CCL39 cells [29], but in contrast with results in NIH 3T3 cells [12] and in macrophages [13]. It is becoming increasingly clear that persistent MAPK activation is necessary for cell proliferation [24,30,31]. Why PC-PLC is mitogenic in some cell types but not in others remains unknown, but is likely to relate to its capability of inducing prolonged MAPK activation. The lack of a mitogenic response in Rat-1 cells is unlikely to be due to insufficient DG levels, since PC-PLC was used at the most effective (non-toxic) dose (Figure 2, upper panel), and the DG generated remained at a 4-fold elevated level for at least 6 h, and metabolic conversion of DG was hardly observed (Figure 1). By comparison, PDGF induced less (3.5-fold) and only transient (2–30 min) formation of DG [31a]

Our finding that PC-PLC (DG) can activate PKC-ζ in the absence of Ras activation does not imply that PDGF-induced PKC-ζ activation is Ras-independent. In fact, it has been shown that PDGF-induced MAPK activation depends on activated Ras [32] and that direct Ras-PKC-ζ interaction/activation is possible [33]. In an entirely different experimental setting, Bjørkøy et al. [34] also found activation of PKC-ζ through PC-PLC. They showed that, in NIH 3T3 cells stably transfected with the *B. cereus* PC-PLC gene, the transformed phenotype of these cells could be reversed with dominant-negative versions of either Raf-1 or PKC-ζ, suggesting that DG activation of both kinases is required for transformation. We are currently investigating whether, in Rat-1 cells, PDGF and PC-PLC activate MAPK through both PKC-ζ and Raf-1 in linear/sequential order or in parallel pathways.

The finding that PC-PLC activates PKC-ζ is unexpected since DG normally fails to activate PKC-ζ, at least *in vitro* [16].

Instead, we have shown that PA is an activator of PKC-ζ *in vitro* (Figure 4) [16], however, we do not see conversion of PC-PLC-generated DG to PA in Rat-1 cells (Figure 1). How then can we explain that DG generation leads to activation of PKC-ζ? One possibility is that activation is indirect; it has been suggested that PC-derived DG may activate an acidic sphingomyelinase [35] that produces ceramide which, in turn, may directly activate PKC-ζ [36,37]. In Rat-1 cells, such a mechanism is unlikely because neither sphingomyelinase nor cell-permeable C-6 ceramide activates the MAPK pathway (Figure 2, upper panel) or PKC-ζ (results not shown). The possibility that the PKC-ζ-MAPK route is activated by PIP_3 through PI3-kinase [38] can be ruled out because wortmannin (0.1–1.0 μM), a selective inhibitor of PI3-kinase [39], failed to block the PC-PLC- or PDGF-induced MAPK cascade (results not shown).

While we still cannot exclude that activation of PKC-ζ by newly formed DG may be indirect, e.g. via an unknown protein kinase, the possibility remains that, *in vivo*, DG can activate PKC-ζ directly in a signalling complex with additional allosteric regulator(s). The mechanisms of activation and intracellular relocation of PKC isotypes appear to be much more complicated than previously thought [40]. That the cysteine-rich region in the regulatory domain of PKC-ζ does not bind phorbol ester [41] does not mean necessarily that it can not bind DG in any conformation. It is known that activation of (conventional) PKC by DG and PMA may not be equivalent, and that binding of one activator may allosterically promote binding of a second activator [42]. *In vivo*, scaffold/adapter/anchor proteins such as 14-3-3 or RACK [4,43], possibly in concert with other kinases/phosphatases may serve as allosteric (co)regulators, in the presence of which DG may act differently on PKC-ζ than in a simple assay *in vitro*. Experimental testing of this possibility may be a challenge for future research.

We thank Henk Hilkmann and José van der Wal for technical assistance. This work was supported by the Dutch Cancer Society.

REFERENCES

1 Seger, R. and Krebs, E. G. (1995) FASEB J. **9**, 726–735
2 Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D. and Rapp, U. R. (1993) Nature (London) **364**, 249–252
3 Marais, R., Light, Y., Paterson, H. F. and Marshall, C. J. (1995) EMBO J. **14**, 3136–3145
4 Aitken, A. (1995) Trends Biochem. Sci. **20**, 95–97
5 Dent, P., Jelinek, T., Morrison, D. K., Weber, M. J. and Sturgill, T. W. (1995) Science **268**, 1902–1906
6 Cai, H., Erhardt, P., Troppmair, J., Diaz-Meco, M. T., Sithanandam, G., Rapp, U. R., Moscat, J. and Cooper, G. M. (1993) Mol. Cell. Biol. **13**, 7645–7651
7 Daum, G., Eisenmann-Tappe, I., Fries, H.-W., Troppmair, J. and Rapp, U. R. (1994) Trends Biochem. Sci. **19**, 474–480
8 Exton, J. H. (1994) Biochim. Biophys. Acta **1212**, 26–42
9 Van Blitterswijk, W. J., Schaap, D. and van der Bend, R. L. (1994) Curr. Topics Membranes **40**, 413–437
10 Ha, K.-S. and Exton, J. H. (1993) J. Biol. Chem. **268**, 10534–10539
11 Carroll, M. P. and May, W. S. (1994) J. Biol. Chem. **269**, 1249–1256
12 Larrodera, P., Cornet, M. E., Diaz-Meco, M. T., Lopez-Barahona, M., Diaz-Laviada, I., Guddal, P. H., Johansen, T. and Moscat, J. (1990) Cell **61**, 1113–1120
13 Xu, X.-X., Tessner, T. G., Rock, C. O. and Jackowski, S. (1993) Mol. Cell. Biol. **13**, 1522–1533
14 Davis, P. D., Hill, C. H., Keech, E., Lawton, G., Nixon, J. S., Sedgwick, A. D., Wadsworth, J., Westmacott, D. and Wilkinson, S. E. (1989) FEBS Lett. **259**, 61–63
15 Schaap, D., van der Wal, J., Howe, L. R., Marshall, C. J. and van Blitterswijk, W. J. (1993) J. Biol. Chem. **268**, 20232–20236
16 Limatola, C., Schaap, D., Moolenaar, W. H. and van Blitterswijk, W. J. (1994) Biochem. J. **304**, 1001–1008
17 Crespo, P., Mischak, H. and Gutkind, J. S. (1995) Biochem. Biophys. Res. Commun. **213**, 266–272
18 Van Blitterswijk, W. J. and Hilkmann, H. (1993) EMBO J. **12**, 2655–2662
19 Burgering, B. M. Th., Medema, R. H., Maassen, J. A., van de Wetering, M. L., van der Eb, A. J., McCormick, F. and Bos, J. L. (1991) EMBO J. **10**, 1103–1109
20 Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F. and Blumberg, P. M. (1993) Mol. Pharmacol. **44**, 298–307
21 Ways, D. K., Cook, P. P., Webster, C. and Parker, P. J. (1992) J. Biol. Chem. **267**, 4799–4805
22 Leevers, S. J. and Marshall, C. J. (1992) Trends Cell Biol. **2**, 283–286
23 Van der Bend, R. L., de Widt, J., Hilkmann, H. and van Blitterswijk, W. J. (1994) J. Biol. Chem. **269**, 4098–4102
24 Meloche, S., Seuwen, K., Pagès, G. and Pouysségur, J. (1992) Mol. Endocrinol. **658**, 845–854
25 Hordijk, P. L., Verlaan, I., van Corven, E. J. and Moolenaar, W. H. (1994) J. Biol. Chem. **269**, 645–651
26 Gordge, P. C. and Ryves, W. J. (1994) Cell. Signal. **6**, 871–882
27 Yeo, E.-J. and Exton, J. H. (1995) J. Biol. Chem. **270**, 3980–3988
28 Berra, E., Diaz-Meco, M. T., Lozano, J., Frutos, S., Municio, M. M., Sánchez, P., Sanz, L. and Moscat, J. (1995) EMBO J. **14**, 6157–6163
29 McKenzie, F. R., Seuwen, K. and Pouysségur, J. (1992) J. Biol. Chem. **267**, 22759–22769
30 Marshall, C. J. (1995) Cell **80**, 179–185
31 Pagès, G., Lenormand, P., L'Allemain, G., Chambard, J.-C., Meloche, S. and Pouysségur, J. (1993) Proc. Natl. Acad. Sci. USA **90**, 8319–8323
31a Van Dijk, M. C. M., Muriana, F. J. G., de Widt, J., Hilkmann, H. and van Blitterswijk, W. J. (1997) J. Biol. Chem. **272**, in the press
32 Burgering, B. M. Th., de Vries-Smits, A. M. M., Medema, R. H., van Weeren, P. C., Tertoolen, L. G. J. and Bos, J. L. (1993) Mol. Cell. Biol. **13**, 7248–7256
33 Diaz-Meco, M. T., Lozano, J., Municio, M. M., Berra, E., Frutos, S., Sanz, L. and Moscat, J. (1994) J. Biol. Chem. **269**, 31706–31710
34 Bjørkøy, G., Øvervatn, A., Diaz-Meco, M. T., Moscat, J. and Johansen, T. (1995) J. Biol. Chem. **270**, 21299–21306
35 Schütze, S., Potthof, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Krönke, M. (1992) Cell **71**, 765–776
36 Lozano, J., Berra, E., Municio, M. M., Diaz-Meco, M. T., Dominguez, I., Sanz, L. and Moscat, J. (1994) J. Biol. Chem. **269**, 19200–19202
37 Müller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D. and Pfizenmaier, K. (1995) EMBO J. **14**, 1961–1969
38 Nakanishi, H., Brewer, K. A. and Exton, J. H. (1993) J. Biol. Chem. **268**, 13–16
39 Ui, M., Okada, T., Hazeki, K. and Hazeki, O. (1995) Trends Biochem. Sci. **20**, 303–307
40 Newton, A. C. (1995) J. Biol. Chem. **270**, 28495–28498
41 Kazanietz, M. G., Bustelo, X. R., Barbacid, M., Kolch, W., Mischak, H., Wong, G., Pettit, G. R., Bruns, J. D. and Blumberg, P. M. (1994) J. Biol. Chem. **269**, 11590–11594
42 Slater, S. J., Ho, C., Kelly, M. B., Larkin, J. D., Taddeo, F. J., Yeager, M. D. and Stubbs, C. D. (1996) J. Biol. Chem. **271**, 4627–4631
43 Mochly-Rosen, D. (1995) Science **268**, 247–251

Received 9 August 1996/17 December 1996; accepted 6 January 1997