Phosphatidic acid activation of protein kinase C- ζ overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids

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Phosphatidic acid (PA) is produced rapidly in agonist-stimulated cells, but the physiological function of this PA is unknown. We have examined the effects of PA on distinct isoforms of protein kinase C (PKC) using a new cell-free assay system. Addition of PA to cytosol from COS cells overexpressing PKC- α , - ϵ or - ζ differentially activated all three isotypes, as shown by PKC autophosphorylation, and prominent phosphorylation of multiple endogenous substrates. In the absence of Ca^{2+} , the diacylglycerol-insensitive ζ -isotype of PKC was most strongly activated by both PA and bisPA, a newly identified product of activated phospholipase D, with each lipid inducing its own profile of protein phosphorylation. BisPA was also a strong activator of PKC- ϵ , but a weak activator of PKC- α . Ca^{2+} , at $\geq 0.1 \mu\text{M}$, inhibited PA and bisPA activation of PKC- ζ , but did not affect

PKC- ϵ activation. In contrast, PKC- α was strongly activated by PA only in the presence of Ca^{2+} . BisPA-induced phosphorylations mediated by PKC- ζ could be mimicked in part by other acidic phospholipids and unsaturated fatty acids. PA activation of PKC- ζ was unique in that PA not only stimulated PKC- ζ -mediated phosphorylation of distinctive substrates, but also caused an upward shift in electrophoretic mobility of PKC- ζ , which was not observed with other acidic lipids or with PKC- α or - ϵ . We have presented evidence that this mobility shift is not caused by PKC- ζ autophosphorylation, but it coincides with physical binding of PA to PKC- ζ . These results suggest that in cells stimulated under conditions where intracellular Ca^{2+} is at (or has returned to) basal level, PA may be a physiological activator of PKC- ζ .

INTRODUCTION

Phosphatidic acid (PA) is rapidly produced in receptor-stimulated cells through activation of phospholipase D (PLD) and/or the concerted action of phospholipase C (PLC) and diacylglycerol (DG) kinase (Billah and Anthes, 1990; Exton, 1990; van Blitterswijk et al., 1991a,b; van der Bend et al., 1992, 1994; Pacini et al., 1993). Several observations suggest that PA may act as a second messenger. For example, in neutrophils, agonist-induced production of PA, rather than DG, closely correlates with secretion and superoxide generation (Cockcroft, 1984; Bauldry et al., 1992); in other cell systems, PA generated by either receptor stimulation or exogenous PLD induces hormone release (Liscovitch and Amsterdam, 1989; Metz and Dunlop, 1990). PA generated by ADP-ribosylation factor-activated PLD is thought to play a role in intracellular membrane traffic (Brown et al., 1993; Kahn et al., 1993; Cockcroft et al., 1994). When added exogenously to cells, PA exhibits growth factor-like activities (Moolenaar et al., 1986; Imagawa et al., 1989; van Corven et al., 1992) and can induce actin polymerization (Ha and Exton, 1993), but cellular targets for PA (either intracellular or at the cell surface) have not been identified to date. Studies *in vitro* indicate that PA can activate several enzymes including PtdIns4P kinase (Moritz et al., 1992), protein kinase C (PKC; Lee and Bell, 1989; Epand et al., 1992; Nakanishi

and Exton, 1992) and a cytosolic protein tyrosine phosphatase (Zhao et al., 1993). Furthermore, Bocckino et al. (1991) have reported that PA can stimulate protein phosphorylation in cytosolic extracts from various rat organs, but the identity of the PA-sensitive kinase(s) remains unknown.

In this study, we have examined the effects of PA on the activity of distinct PKC isotypes using a COS cell expression system. The PKC family consists of at least ten different isozymes (Stabel and Parker, 1991; Nishizuka, 1992), which can be divided into three distinct subgroups on the basis of their biochemical and structural properties: (1) the 'classical' isoforms, regulated by Ca^{2+} and DG plus phosphatidylserine (PS), comprising PKC- α , - β_1 , - β_{II} and - γ ; (2) the 'new' isoforms PKC- ϵ , - δ , - η and - θ , which lack the Ca^{2+} -binding domain; and (3) the 'atypical' isoforms PKC- ζ , - ι and - μ , which differ from the others in that they are Ca^{2+} - and DG-independent and refractory to phorbol ester activation (Ono et al., 1989; Gschwendt et al., 1992; Liyanage et al., 1992; Ways et al., 1992; Nakanishi and Exton, 1992; Selbie et al., 1993; Johannes et al., 1994). The physiological activator(s) of the last PKC isotypes have not been identified, although *in vitro* studies suggest that purified PKC- ζ can be activated by PtdIns(3,4,5) P_3 (Nakanishi et al., 1993).

In previous studies on PKC activation, workers have incubated purified PKCs with various lipids and artificial substrates. In our present study we have utilized cytosols prepared from COS cells

Abbreviations used: PA, phosphatidic acid; PKC, protein kinase C; PLD, phospholipase D; PLC, phospholipase C; DG, diacylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; ECL, Enhanced ChemiLuminescence System; DMEM, Dulbecco's modified Eagle's medium; DOC, sodium deoxycholate; NRS, normal rabbit serum; Staph A, *Staphylococcus aureus* (COWAN I strain) bacteria; LPA, lysophosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin; PE, phosphatidylethanolamine.

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that overexpress either PKC- ζ , PKC- α or PKC- ϵ , being representatives of the three PKC subclasses. This cell-free system greatly facilitates the detection of PKC-mediated phosphorylations of endogenous proteins against a relatively low background. We compared the effect of PA with that of other acidic lipids, including bisphosphatidic acid (bisPA), a newly identified product of the simultaneous action of PLD and PLC in receptor-stimulated cells (van Blitterswijk and Hilkmann, 1993). We report that PA, bisPA and various other acidic lipids, in the absence of Ca^{2+} , can differentially activate PKC- ζ , as revealed by PKC autophosphorylation, and that each lipid induces its own characteristic pattern of phosphorylated substrates. However, PA is unique with respect to its induced phosphorylation pattern and in that it induces an upward mobility shift of PKC- ζ on SDS polyacrylamide gel. We propose that PA is a candidate physiological activator of PKC- ζ .

EXPERIMENTAL

Materials

Dioleoyl-PS and phosphatidylinositol (PI) were obtained from Avanti Polar Lipids; bisPA (tetraoleoyl-) and arachidonic acid from Serdary Research Laboratories (London, Ontario, Canada); PA (prepared from egg yolk phosphatidylcholine), all other lipids, BSA (fraction V; fatty acid free), phenylmethanesulphonyl fluoride, leupeptin and aprotinin were from Sigma; [γ - ^{32}P]ATP and Enhanced ChemiLuminescence System (ECL) from Amersham; acrylamide and *N,N'*-methylenebisacrylamide from Merck (Darmstadt, Germany); nitrocellulose paper from Schleicher and Schuell (Dassel, Germany); Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum from Flow Laboratories; peroxidase-conjugated rabbit anti-mouse IgG from DAKO-Immunoglobulins (Denmark). The PKC inhibitor Ro 31-8220 (compound 3 from Davis et al., 1989) was kindly provided by Roche Research Center (Welwyn Garden City; U.K.).

Antibodies

Antibodies against PKC- α , - ϵ , - ζ and *c-raf-1* were raised in rabbits, and were directed against oligopeptides based on the C-termini of these proteins (Schultz et al., 1985; Marais and Parker, 1989; Schaap et al., 1989; Ways et al., 1992).

cDNA constructs

pmtM is a pMT-2-derived COS cell expression vector that contains a multiple cloning region (Gebbink et al., 1991); pmtSM is derived from pmtM and contains three upstream stop codons in all three reading frames (to be described elsewhere). pmtM-PKC- α contains bovine PKC- α (Schaap et al., 1993). pMT2-PKC- ϵ contains mouse PKC- ϵ (Schaap et al., 1989); rat PKC- ζ cDNA (Ways et al., 1992) was subcloned in pmtSM as an *XbaI/XbaI* fragment. Human *c-raf-1* cDNA was subcloned in pmtM as an *SmaI/XbaI* fragment.

Cell culture, transfection, and preparation of cytosol

COS M6 cells (kindly provided by J. Schlessinger) were grown in DMEM supplemented with 10% foetal calf serum and containing penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), 2 mM L-glutamine. For transfection, 1.5×10^5 cells were seeded into 6 cm dishes and 7 μg of plasmid DNA per plate was used following the calcium phosphate precipitation protocol. To minimize differences in protein expression, the same expression vector was used for all PKC isoforms. Twenty-four hours after transfection, cells

were put into serum-free DMEM and, after an additional 24 h, were used for the experiments. Transfected cells were scraped off the plates in 250 μl of Hepes buffer (5 mM, pH 7.5), containing 250 mM sucrose, 2 mM EGTA, 1 mM MgCl_2 , 80 μM leupeptin and 20 μM aprotinin, sonicated for 10 s on ice with a probe sonicator (Branson) and centrifuged for 60 min at 100 000 *g* in an airfuge (Beckman). The supernatant (cytosolic fraction) was used for experiments.

Lipid preparations

Chloroform/methanol-dissolved lipids were dried under a stream of N_2 , resuspended in a solution containing fatty acid-free BSA (1 mg/ml) and sonicated for 5 min on ice with a probe sonicator (Branson). ^{32}P -labelled PA was prepared as follows: 2 mg of 1,2-dioleoyl-*sn*-glycerol was incubated for 2 h at 37 °C with 1 mg of partially purified human DG kinase (Schaap et al., 1990) in 1 ml (total volume) of buffer containing 50 mM Tris/HCl, pH 7.4, 10 mM MgCl_2 , 10 mM NaF, 1 mM sodium deoxycholate (DOC), 1.8 μM [γ - ^{32}P]ATP. Lipids were extracted according to Bligh and Dyer (1959) in the presence of 0.1 M acetic acid, separated by t.l.c. using chloroform/methanol/acetic acid/water (50:30:8:4) and PA was extracted from the silica gel. Specific activity of [^{32}P]PA was 171 Ci/mmol.

Kinase assay

Cytosolic fractions (10 μl containing 10 μg of protein) from transfected COS cells were mixed with 35 μl of a buffer containing 50 mM Hepes, pH 7.2, 10 mM MgCl_2 , 2 mM EGTA, 80 μM leupeptin, 20 μM aprotinin, 3 μCi of [γ - ^{32}P]ATP (3000 Ci/mmol), and 5 μl of a 10-fold concentrated suspension of lipids or vehicle (0.01% BSA, final concn.) in the control. After 5 min incubation at room temperature the reaction was stopped by adding SDS sample buffer (Laemmli, 1970). Samples were boiled for 5 min and analysed by SDS gel electrophoresis. Gels were dried and exposed, usually overnight at -70 °C, using X-OMAT AR film (Kodak) with intensifying screen.

Immunoprecipitation

Kinase reactions were blocked by addition of 10 vol. of ice-cold RIPA buffer (50 mM Tris/HCl, pH 7.5/150 mM NaCl/1% Triton X-100/0.1% SDS/1% deoxycholate/2 mM EDTA) containing 20 μM leupeptin, 20 μM aprotinin, and 1 mM phenylmethanesulphonyl fluoride. Samples were precleared by incubation for 15 min at 4 °C, with a 10% solution (in RIPA buffer) of formalin-fixed heat-killed *Staphylococcus aureus* (COWAN I strain) bacteria (Staph A) precoated with normal rabbit serum (NRS), followed by two incubations for 15 min at 4 °C with Staph A. Incubation with antibodies were performed overnight at 4 °C. Immunocomplexes were then incubated for 1 h with Staph A, and the precipitate was washed six times with RIPA buffer. Precipitates were boiled for 5 min in SDS sample buffer and analysed on SDS 10% polyacrylamide gel. Dried gels were exposed at -70 °C for 2-7 days to X-OMAT AR film (Kodak) with one intensifying screen. Immunoprecipitated proteins were excised from the gel and their radioactivity was measured by Čerenkov counting.

Immunoblot analysis

Cytosols from transfected COS cells were mixed (1:1) with 2 $\times$ SDS sample buffer and boiled for 5 min. Samples (10-20 μg of proteins) were analysed on 10% SDS polyacrylamide gel, and electrophoretically transferred to nitrocellulose paper at 4 °C for 2 h. Blots were incubated for 1 h with 5% defatted dry milk in

PBS containing 0.1% Triton X-100 to block non-specific binding sites, and then incubated overnight with the anti-peptide antisera. Antisera were diluted 1:2000 for *c-raf-1*, PKC- α and PKC- ϵ and 1:5000 for PKC- ζ . Immunoreactivity was detected by ECL (Amersham).

Immunodepletion

The kinase reaction was stopped after 5 min by adding ice-cold RIPA buffer, and samples were precleared as described for the immunoprecipitation experiments. A portion of these samples was mixed with SDS sample buffer and the remainder was incubated with antiserum against PKC- ζ or NRS. The immunocomplexes were precipitated with Staph A and processed as described for the immunoprecipitation. The supernatants were mixed with SDS sample buffer and analysed on SDS gel (10% polyacrylamide). Gels were dried and exposed for 3 h at -70°C to X-OMAT AR film (Kodak) with intensifying screen.

Binding assay

Chloroform/methanol-dissolved [^{32}P]PA (1.2 μCi) was dried under a stream of N_2 , mixed with a 500-fold excess of cold PA to a final concentration of 50 $\mu\text{g}/\text{ml}$, and further sonicated for 5 min in a bath sonicator. This lipid preparation was mixed with cytosols (50 μg total protein) from transfected COS cells, incubated for 5 min at room temperature and analysed on 7.5% SDS gel. The gel was dried and exposed for 3–7 days at -70°C to X-OMAT AR film (Kodak) with one or two intensifying screens.

Determination of free Ca^{2+}

A computer program (Chelate) was used to determine the free Ca^{2+} concentration in Ca^{2+} /EGTA buffer.

RESULTS

To search for PA-dependent protein kinase activity, we initially performed experiments with extracts from Rat-1 cells. We observed PA-dependent protein phosphorylation in the cytosolic but not in the particulate fraction (results not shown), reminiscent of the results obtained by Bocchino et al. (1991) who used cytosolic extracts from various rat tissues. However, the PA-induced phosphoprotein profile in Rat-1 cytosols was quite variable among different experiments, and its analysis was hampered by a relatively high background phosphorylation level. We therefore turned to the COS cell expression system, in which selected PKC isoforms were overexpressed, resulting not only in amplification of PKC activity but also in better reproducible phosphoprotein patterns induced by PA and other lipids against a relatively low background.

PA-induced phosphorylation of endogenous substrates in COS cells transfected with PKC isoforms

Typical members of the three subclasses of PKC, the α -, ϵ - and ζ -isoforms, were overexpressed in COS cells and cytosolic phosphorylation patterns induced by PA were compared with those induced by PS plus DG, the classical PKC-activating lipids (Figure 1). [Although PKC- ζ is insensitive to DG (see Figure 3), we used the PS/DG mixture also for PKC- ζ , to compare the three PKC isozymes under identical physicochemical conditions.] Addition of PA to cytosols of control cells (transfected by empty vector pmtSM) resulted in phosphorylation of a number of endogenous substrates (Figure 1a). The PA-induced phosphorylation pattern was different from that evoked by PS/DG (Figure 1a), suggesting that the PA-dependent phosphorylations were

Figure 1 Lipid-induced phosphorylation of endogenous substrates in cytosol from transfected COS cells

COS M6 cells were transfected with pmtSM, pmtM-PKC- α , pmt2-PKC- ϵ or pmtSM-PKC- ζ (as indicated) and cytosols were prepared as described in the Materials and methods section. Equal amounts of cytosolic protein (10 μg) were incubated for 5 min in the presence or the absence (control, C) of 100 $\mu\text{g}/\text{ml}$ of the lipids indicated, in buffer containing 3 μCi of [γ - ^{32}P]ATP as described in the Materials and methods section. (Note that although PKC- ζ is insensitive to DG, we used the PS/DG mixture also for PKC- ζ to compare the three PKC isozymes under identical physicochemical conditions). For pmtM-PKC- α -transfected cells, 1 μM free Ca^{2+} was present where indicated. Phosphorylated proteins were analysed on a 10–15% gradient SDS polyacrylamide gel and autoradiographs were exposed for 2 days (a) or 5 h (b). Arrows indicate the position of proteins of molecular masses of 27, 52 and 78 kDa, as described in the text. Western blots of individual transfectants showed high expression levels of all protein kinase cDNAs transfected (results not shown). Similar results were obtained in three separate experiments. Molecular mass markers (kDa) are indicated.

not mediated merely by the classical PKCs. In cells overexpressing PKC- α , ϵ or ζ , a 10- to 50-fold increase in lipid-induced protein phosphorylation was observed for all three isoenzymes tested (Figure 1b). In PKC- α -overexpressing cells, most of the PA-induced phosphorylation was found to depend on the presence of free Ca^{2+} (1 μM), as expected on the basis of the known Ca^{2+} dependency of this PKC isotype. Under these conditions, PA was capable of stimulating PKC- α -mediated phosphorylations as effectively as PS/DG. In cells overexpressing the PKC- ϵ and ζ isoforms, PA induced a more prominent phosphorylation pattern than PS/DG (Figure 1b). It was evident that the three distinct PKC isoforms differed in their substrate specificity (Figure 1b and results not shown). For example, proteins of 27 and 52 kDa were phosphorylated following addition of PA to PKC- ζ - and PKC- α -containing cytosol (the latter only in the presence of Ca^{2+}); these proteins were not phosphorylated by PKC- ϵ . Furthermore, PKC- ζ -transfected cells contained a major phosphoprotein of 78 kDa, which represented the autophosphorylated form of PKC- ζ , as will be shown below.

To test the specificity of PA-induced phosphorylation by PKCs, we also examined the effect of PA on the *c-raf-1* protein kinase. This kinase is structurally related to PKC- ζ in that it contains a single conserved cysteine repeat in its regulatory domain (Stabel and Parker, 1991). However, PA failed to stimulate protein phosphorylation in cytosol from *c-raf-1*-overexpressing COS cells (results not shown).

PA-induced autophosphorylation of PKCs

PKCs can undergo autophosphorylation via an intramolecular reaction (Hannun et al., 1985; Newton and Koshland, 1987),

Figure 2 Lipid-induced autophosphorylation of PKC isotypes detected in immunoprecipitates

Cytosolic protein (10 μ g) from COS cells transfected with either PKC- α , PKC- ϵ or PKC- ζ was incubated with 50 μ M [γ - 32 P]ATP (15 μ Ci) for 5 min, in the presence or absence of 100 μ g/ml lipids, in either Ca^{2+} -free medium or 1 μ M Ca^{2+} (as indicated). Note that although PKC- ζ is insensitive to DG, we used the PS/DG mixture also for PKC- ζ to compare the three PKC isozymes under identical physicochemical conditions. PKCs were immunoprecipitated with the corresponding antisera, as described in the Materials and methods section, with a recovery of roughly 80%. Immunoprecipitates were then analysed on 10% SDS polyacrylamide mini-gels. Only regions of interest are shown. Radioactivity associated with immunoprecipitated PKC- ζ (c.p.m.): control (C), 40; PA, 140; PS/DG, 108. Corresponding c.p.m. values for immunoprecipitated PKC- α (+ Ca^{2+}) are C, 25; PA, 118; PS/DG, 46. Similar results were obtained in three separate experiments. We note that immunoprecipitates also contained a proteolytic PKC product of about 55 kDa (results not shown).

Figure 3 PKC- ζ activation by various lipids

Cytosols from COS cells transfected with pmtSM-PKC- ζ were incubated for 5 min in the presence of the indicated lipids in buffer containing 3 μ Ci of [γ - 32 P]ATP, as described in the Materials and methods section. C, control without added lipid. Final concentrations of the various lipids: 50 μ M for palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), and arachidonic acid (20:4); 22 μ M for bisPA; 140 μ M for PA; 214 μ M for LPA and about 120 μ M for PI, PG, PE, PC, PS, PS/DG (5:1 molar ratio), CL and ceramide (CER). DOC was used at a final concentration of 100 μ M. Substrate phosphorylation including PKC- ζ autophosphorylation (78 kDa band) was analysed on a 10–15% gradient SDS polyacrylamide gel. Similar results were obtained in three separate experiments. Molecular mass markers are indicated (kDa).

which is considered to be a reliable measure of PKC activation (Mitchell et al., 1989). Figure 2 shows autophosphorylation of the various PKCs in COS cytosols treated with either PA or PS/DG, followed by immunoprecipitation. In the nominal absence of free Ca^{2+} , PKC- α shows hardly any lipid-induced

autophosphorylation. However, in the presence of 1 μ M free Ca^{2+} , PKC- α undergoes significant autophosphorylation, which is further increased by addition of PA but much less by PS plus DG (4.7-fold versus 1.8-fold above control, respectively). Figure 2 also shows that autophosphorylation of PKC- ϵ is more efficiently induced by PA than by PS/DG. PKC- ζ is already phosphorylated in untreated cytosol and the addition of PA stimulates this autophosphorylation of PKC- ζ slightly more than that induced by PS (3.2-fold versus 2.5-fold, respectively). We note that the degree of PKC autophosphorylation correlates well with phosphorylation of endogenous substrates (cf. Figures 2 and 1).

PKC- ζ activation by PA, bisPA and other acidic lipids

Using artificial substrates, Nakanishi and Exton (1992) reported that various acidic lipids can activate purified PKC- ζ *in vitro*. We used cytosols from transfected COS cells to test whether acidic lipids other than PA, in particular the novel phospholipid bisPA, could induce PKC- ζ -mediated phosphorylation of endogenous substrates. Figure 3 shows that bisPA, PS (with or without DG), lysoPA (LPA), PI, phosphatidylglycerol (PG), cardiolipin (CL) and *cis*-unsaturated fatty acids induce phosphorylation of a number of endogenous substrates to different degrees. BisPA consistently induces the strongest substrate phosphorylation pattern, at an even lower concentration than for other lipids tested. The most prominent PKC- ζ substrates are proteins of apparent molecular masses of 27, 30, 52, 58 and 78 kDa. The last protein is PKC- ζ itself, as will be shown below. Phosphorylation of the 27 kDa protein is consistently much more pronounced with PA as an activator. Figure 3 further shows that *cis*-unsaturated fatty acids, notably oleic acid (18:1) and arachidonic acid (20:4), can activate PKC- ζ , whereas the saturated ones such as stearic (18:0) and palmitic acid (16:0) are ineffective, even when added at very high concentrations (results not shown). This result agrees, at least qualitatively, with the *in vitro* data of Nakanishi and Exton (1992), who used purified PKC- ζ . Importantly, it is seen that DG fails to activate PKC- ζ (Figure 3), a result in agreement with previous reports (see the Introduction section); phorbol ester (results not shown), ceramide, PC and phosphatidylethanolamine (PE) are also ineffective. Finally, deoxycholate (DOC) fails to mimic the action of PA and bisPA, which argues against a non-specific detergent-like effect of the lipids tested.

Figure 4 illustrates the PA and bisPA dose dependencies of PKC- ζ -mediated phosphorylations. On a molar basis, bisPA is found to be maximally active at a 3- to 6-fold lower concentration than PA in triggering PKC- ζ -mediated protein phosphorylation. Figure 4 also emphasizes the differential substrate preference imposed by PA and bisPA; for example, compare the 27 and 52 kDa phosphoproteins induced by PA with the 30 and 58 kDa phosphoproteins induced by bisPA. BisPA is also a strong activator of PKC- ϵ -mediated phosphorylation, particularly of the 30 kDa substrate, but is only a weak activator of PKC- α (results not shown).

Ca^{2+} inhibits PKC- ζ activation

Since Ca^{2+} is essential for PA activation of PKC- α (Figures 1 and 2), we thought this was likely to be due to the occurrence of the Ca^{2+} binding (C2) domain in the primary structure of this isotype. In agreement with this notion, activation of purified PKC- ϵ or - ζ , which both lack such a Ca^{2+} binding domain, was previously found to be independent of Ca^{2+} or even inhibited at millimolar Ca^{2+} concentrations (Schaap and Parker, 1990; Nakanishi and Exton, 1992). In the COS cell lysates, we found

Figure 4 Dose-dependent activation of PKC- ζ by PA and bisPA

Cytosols from PKC- ζ -transfected COS cells were incubated for 5 min in the presence of increasing amounts of PA or bisPA, in buffer containing 3 μ Ci of [γ - 32 P]ATP, as described in the Materials and methods section. Phosphorylated proteins were analysed on a 10–15% gradient SDS polyacrylamide gel. The arrow indicates the position of PKC- ζ (78 kDa).

Figure 5 Ca^{2+} dose-dependently inhibits PA and bisPA activation of PKC- ζ

Cytosols from PKC- ζ -transfected COS cells were incubated for 5 min in buffer containing 3 μ Ci of [γ - 32 P]ATP without (control) or with PA (30 μ g/ml) or bisPA (10 μ g/ml), in the presence of increasing amounts of free Ca^{2+} , as indicated. Phosphorylated proteins were analysed on a 10–15% gradient SDS polyacrylamide gel. The arrow indicates the position of PKC- ζ (78 kDa). Similar results were obtained in three different experiments.

that PA- (and bisPA)-activation of PKC- ϵ is also independent of Ca^{2+} (results not shown). Unexpectedly, however, PA or bisPA activation of PKC- ζ in this cell-free system is inhibited by Ca^{2+} (at ≥ 0.1 μ M) in a dose-dependent fashion (Figure 5). Apparently, the cell-free system differs in this respect from an assay with purified PKC- ζ and an artificial substrate, peptide ϵ (Nakanishi and Exton, 1992).

Figure 6 Immunodepletion of the 78 kDa protein from cytosol of PKC- ζ -transfected COS cells

Cytosol was incubated for 5 min with 100 μ g/ml PA, in the presence of 3 μ Ci of [γ - 32 P]ATP. Cytosols were divided into two and either left untreated (–) or immunodepleted (+) with PKC- ζ -specific antiserum or NRS and then analysed on 10% SDS polyacrylamide mini-gels. The immunoprecipitate (IP) is shown on the right. Similar results were obtained in a separate experiment. Molecular mass markers are indicated (kDa).

PA, but not other lipids, induces an electrophoretic mobility shift of PKC- ζ

Figure 4 shows that in PKC- ζ -overexpressing cells, PA-induced phosphorylation of the 78 kDa protein is accompanied by a reduced electrophoretic mobility (to 80–82 kDa apparent molecular mass) in a dose-dependent fashion. Such a mobility shift is not seen with bisPA or any other lipid, even at the highest concentrations tested (Figures 3 and 4). This 78 kDa protein is PKC- ζ itself, as revealed by immunodepletion experiments (Figure 6). Moreover, this protein band is not detected in cells transfected with either empty vector or other PKC isoforms (Figure 1 and results not shown). A plausible explanation for the PA-induced mobility shift of PKC- ζ would be its auto-phosphorylation. We have examined this possibility using a highly selective PKC inhibitor, Ro 31-8220 (Davis et al., 1989; Walker and Watson, 1993). Ro 31-8220 (3 μ M) effectively inhibits PKC- ζ -mediated phosphorylation of endogenous substrates (Figure 7a) as well as PKC- ζ autophosphorylation (as seen in PKC- ζ immunoprecipitates; Figure 7b), although some weak PA-induced autophosphorylation still remains visible. Despite the dramatic reduction of PKC- ζ autophosphorylation in the presence of Ro 31-8220, the PA-induced gel-shift of PKC- ζ is still present, as analysed by immunoblotting (Figure 7c). In contrast, no shift is observed when PA is added to PKC- α or - ϵ -containing cytosols (Figure 7d). The Western blot (Figure 7c) further shows that PKC- ζ protein levels are virtually the same in all samples, implying that the dramatic reduction in radioactivity recovered in immunoprecipitates of Ro 31-8220-treated samples (first two lanes of Figure 7b) is entirely due to inhibition of kinase activity. It is not likely that the weak PA-induced autophosphorylation of PKC- ζ , that remains in the presence of Ro 31-8220, is sufficient to cause the electrophoretic mobility shift, because the intensity of phosphorylation in this shifted band on gel is less than in the control without Ro 31-8220. These results demonstrate that the observed mobility shift of PKC- ζ cannot be explained by autophosphorylation.

Binding of [32 P]PA to PKC- ζ

We next examined whether the PKC- ζ mobility shift was caused by direct binding of PA to PKC- ζ . Cytosols from PKC-

Figure 7 PA-induced electrophoretic mobility shift of PKC- ζ

Effect of the PKC inhibitor Ro 31-8220 on PKC- ζ -mediated phosphorylation of COS cytosolic proteins (a), on lipid-induced autophosphorylation of PKC- ζ (immunoprecipitation) (b), and on the mobility of immunoblotted proteins of the same samples (c). (d) Immunoblot analysis of PA-treated cytosols from PKC- α and - ϵ -transfected COS cells. Cytosols from PKC-transfected cells were incubated for 5 min with the indicated lipids (30 μ g bisPA/ml and 100 μ g/ml for the others) in the presence of 3 μ Ci (a) or 15 μ Ci of [γ - 32 P]ATP (b, c). Where indicated, 3 μ M Ro 31-8220 or the corresponding amount of the vehicle (dimethyl sulphoxide) were included in the assay. C, control (no lipids added). The arrow in (a) indicates the position of PKC- ζ . Note that although PKC- ζ is insensitive to DG, we stimulated with PS/DG mixture instead of PS to obtain comparable physicochemical conditions as in Figures 1 and 2. Similar results were obtained in three separate experiments.

Figure 8 32 P-labelled PA binding to PKC- ζ

Cytosols from pmtSM- (control), PKC- α -, PKC- ϵ - or PKC- ζ -transfected COS cells were incubated in the presence of 50 μ g/ml [32 P]PA (0.342 Ci/mmol) for 5 min. Samples were then boiled for 5 min in SDS sample buffer and analysed on 7.5% SDS gel. Arrows show the predicted positions of the PKC isoforms.

transfected COS cells were incubated with 32 P-labelled PA and then analysed by SDS/PAGE and autoradiography. Figure 8 shows that, despite the harsh denaturing conditions of hot SDS sample buffer, [32 P]PA remained associated to a protein of about 80 kDa that was present in the cytosol from PKC- ζ -transfected cells, but not in cytosols from pmtSM-, PKC- α - or PKC- ϵ -transfected cells. This radiolabelled protein co-migrated with the PKC- ζ band that was detectable on Coomassie Blue-stained gel

(results not shown). These results demonstrated that PA strongly binds to PKC- ζ , although we note also some binding of [32 P]PA to other cytosolic proteins, including a protein co-migrating with Coomassie Blue-stained PKC- α (in PKC- α -transfected cells) (Figure 8). Collectively, the data allow us to conclude that PA binds to and activates PKC- ζ and that, most likely, PA binding is responsible for the observed electrophoretic mobility shift.

DISCUSSION

In this paper we have explored the potential second messenger role of PA by testing its ability to activate distinct PKC isotypes in cytosolic extracts from transfected COS cells. A major advantage of the system is that it allows significant amplification of phosphorylation mediated by the transfected protein kinases and that it does not need to rely on the use of artificial substrates but, instead, uses endogenous cytosolic proteins as substrates. The main results of the present study can be summarized as follows. First, PA binds to and activates PKC- ζ , resulting in a reduced electrophoretic mobility of the enzyme and in selective phosphorylation of cytosolic substrates (27 and 52 kDa). We should note that PA not only binds to PKC- ζ , but also to PKC- α and serum albumin (results not shown). This binding appears strong enough to be resistant not only to detergents but also to boiling SDS sample buffer. Such tight lipid-protein interactions that resist SDS treatment have also been found between PtdIns(4,5) P_2 and α -actinin (Fukami et al., 1992) and for the binding of lysoPA to serum albumin (Tigyi et al., 1990). Second, other acidic lipids, including newly discovered bisPA, are also capable of activating PKC- ζ but impose upon the enzyme a different substrate selectivity (cf. PA- versus bisPA-stimulated PKC- ζ ; Figures 4 and 5). Third, PA can also activate other PKC isotypes, albeit with different efficiency and depending differentially on Ca^{2+} . Elevation of Ca^{2+} levels significantly inhibits PA (and bisPA) activation of PKC- ζ but not of PKC- ϵ , while, in contrast, it stimulates PKC- α activation. Taken together, the results indicate that, in a cell-free system, PA activates PKC- ζ in a unique fashion, which raises the likelihood that PA may also play a unique role *in vivo* by functioning as a second messenger to activate PKC- ζ in response to receptor stimulation. This seems very well possible when considering the effective concentration range of PA (3–30 μ g/ml; Figure 4) and assuming enhanced PA concentrations of 360 μ g/ml in agonist-stimulated cells, as estimated by Bocchino et al. (1991).

PKC- ζ is ubiquitously expressed, but very little is known about its normal physiological function. Recent evidence suggests that PKC- ζ has a critical but as yet undefined role in mitogenic signalling (Berra et al., 1993). This raises the possibility that the mitogenic action in fibroblasts of exogenous PA or bacterial PLD (Moolenaar et al., 1986; Fukami and Takenawa, 1992; van Corven et al., 1992) may be explained by PA activation of PKC- ζ . To this end, exogenous PA must be incorporated in monomeric form in the outer leaflet of the plasma membrane lipid bilayer and undergo transbilayer diffusion ('flipping') (Tokumura et al., 1992) or be internalized into cells by some other mechanism. Of note is that intracellular accumulation of PA, rather than an increase in DG, correlates well with mitogenesis in growth factor-stimulated fibroblasts (Fukami and Takenawa, 1992).

The classical PKC isotypes are activated by DG and Ca^{2+} in the presence of PS. However, several *in vitro* studies have shown that other lipids may also participate in PKC activation, including phosphoinositides, PA, CL, PG, *cis*-unsaturated fatty acids and lyso-PC (McPhail et al., 1984; Hannun et al., 1986; Oishi et al., 1988; Lee and Bell, 1989; Schaap and Parker, 1990; Shinomura et al., 1991; Nakanishi and Exton, 1992; Nishizuka, 1992; Orr

and Newton, 1992; Saido et al., 1992; Khan et al., 1993; Kochs et al., 1993; Nakanishi et al., 1993). These findings raise questions about the physiological relevance of these multiple activators. Since some of these compounds are produced at different time points during cell stimulation, it has been hypothesized that they may keep PKC active for a prolonged time period, which might be essential for long-term responses, such as cell proliferation and differentiation (Nishizuka, 1992). Alternatively, distinct PKC isotypes may be differentially regulated by lipids (Hug and Sarre, 1993; Khan et al., 1993; Nakanishi et al., 1993) and may thus operate in different signal transduction pathways.

In COS cell cytosols at the appropriate free Ca^{2+} content, we have observed a marked PA-induced autophosphorylation of each isotype, more pronounced than that induced by PS/DG. Being an intrapeptide reaction, autophosphorylation is a strong indication that PA activates the enzyme directly. Protein kinase activation by PA appears to be specific for PKCs, since PA has failed to activate the structurally related Ser/Thr kinase *c-raf-1*.

Our result that PA, like DG plus PS, can activate PKC- α only in the presence of Ca^{2+} is in agreement with results of Epand et al. (1992), who have used artificial substrates. In contrast to PKC- α , we found that PA activation of PKC- ζ is inhibited by Ca^{2+} . This is an unexpected result, since Nakanishi and Exton (1992) did not find such inhibition using purified PKC- ζ and an artificial substrate, peptide ϵ . The reason for this apparent discrepancy is unclear. It may be due to a Ca^{2+} -regulated factor (protein kinase/phosphatase?) that is present in COS cytosol but absent in the *in vitro* assay with purified PKC- ζ . This would imply that in intact stimulated cells, no substantial PKC- ζ activation will be induced by PA (or bisPA) when intracellular Ca^{2+} is elevated. Conversely, when Ca^{2+} remains at, or returns to, basal level, PKC- ζ may possibly become activated by the DG kinase/PLD products PA/bisPA, while PKC- α activation will concomitantly be suppressed.

A study *in vitro* by Nakanishi et al. (1993) has suggested that PKC- ζ may be a target for $\text{PtdIns}(3,4,5)\text{P}_3$, a novel phosphoinositide implicated in growth factor action. Since the experimental conditions in this study are different, we cannot compare the specificity of this lipid with that of PA/bisPA in the activation of PKC- ζ . In a future study, it would be interesting to directly compare these lipids in the present COS cell system. It is relevant to note here that other PKCs can also be activated by $\text{PtdIns}(3,4,5)\text{P}_3$ *in vitro* (Singh et al., 1993).

That PKC- ζ is activated by other acidic lipids as well (Figure 3) is consistent with previous data obtained with purified PKC- ζ (Nakanishi and Exton, 1992). Of special interest are the effects of the novel phospholipid bisPA. BisPA is rapidly generated in stimulated fibroblasts as the product of a PLD-mediated transphosphatidyl reaction that uses DG as the acceptor for the phosphatidyl moiety (van Blitterswijk and Hilkmann, 1993). No physiological function has yet been ascribed to this uncommon phospholipid. In the present experiments, bisPA is the most potent PKC- ζ activator of all lipids tested (in the absence of Ca^{2+}). However, bisPA does not cause the electrophoretic mobility shift of PKC- ζ found with PA, and it stimulates PKC- ϵ activity as well, independent of Ca^{2+} (results not shown). Further studies will be necessary to assess the physiological relevance of bisPA in signal transduction.

We observed that different activating lipids impose upon PKC- ζ a different pattern of substrate phosphorylations. It is not clear whether this represents a direct effect of the lipids on the enzyme itself, on the substrate, or on both. Pears et al. (1991) have demonstrated that the regulatory domain of PKC is involved in substrate selection. It is only one step further to suggest that, via the regulatory domain, different activators may also play a

role in substrate specificity. Newton and Koshland (1990) have shown *in vitro* that substrate specificity of PKC is affected by the PS concentration in membranes and discuss this in terms of different affinities of particular substrates to negatively charged membranes. It may therefore well be that in intact cells, such interactions with anionic (phospho)lipids determine substrate presentation to PKC isotypes.

Isoform-dependent differences in substrate phosphorylation have, so far, been described mainly for artificial substrates (Hug and Sarre, 1993). Our results that at least two substrates, of 27 kDa and 52 kDa, are phosphorylated by PA-stimulated PKC- ζ and - α (the latter only with Ca^{2+}) but not by PKC- ϵ (Figure 1), are one of the few demonstrations of a difference in endogenous substrate preference among PKC isoforms (Majumdar et al., 1991; Nishikawa et al., 1992).

In conclusion, our results demonstrate a unique interaction between PA and PKC- ζ , which is not found for other PKC isotypes or for the other PKC- ζ activators tested; PA activates PKC- ζ , as inferred from its autophosphorylation, and evokes a unique pattern of phosphorylated substrates, the identification of which should help to clarify the role of PKC- ζ in cellular signalling. PA-activated PKC- ζ may provide the link between receptor-stimulated PLD and the cellular actions of PA. Thus, PA must be considered as a candidate physiological activator of PKC- ζ .

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