Activation of purified human protein kinase C α and β I isoenzymes *in vitro* by Ca^{2+} , phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate

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The increasing number of eukaryotic protein kinase C (PKC) isoenzymes which have been described has raised great interest in potential differences in the cellular expression, the mode of activation and the substrate specificity of these isoenzymes. The last two aspects have mostly been studied with isoenzymes purified from rat or bovine brain or from recombinant-baculovirus-infected insect cells. In this study, we have expressed the human PKC isoenzymes α and β I in recombinant-baculovirus-infected insect cells. The isoenzymes were purified to homogeneity by a four-step procedure which included a reversible Ca^{2+} -dependent association/dissociation to and from the endogenous membranes of the lysed insect cells. Characterization of the purified enzymes with respect to ATP requirement and substrate specificity, using the epidermal-growth-factor receptor peptide and histone III-S respectively, revealed no isoenzyme-specific

differences. Activation by trypsin or Ca^{2+} and a variety of different phospholipids and phosphoinositides (in a mixed-micellar assay) gave the following results. Proteolytic cleavage of the PKC isoenzymes by trypsin generated fully activated phospholipid-independent PKC β I, whereas PKC α reached only 50 % of the activity obtained in the presence of phospholipids. PKC α and β I showed no difference in their dependence on Ca^{2+} , diacylglycerol (DAG) and phosphatidylserine (PS). Replacement of either DAG or PS by phosphatidylglycerol, cardiolipin, phosphatidylcholine and several phosphoinositides revealed that $\text{PtdIns}(4,5)\text{P}_2$ can act as a PKC activator similar to DAG, whereas PtdIns can substitute for PS as a cofactor of activation. Thus, at least for the PKC isoenzymes α and β I, a combination of PtdIns and $\text{PtdIns}(4,5)\text{P}_2$ can fully replace PS and DAG *in vitro* as the classical activators of PKC.

INTRODUCTION

The protein kinase C (PKC) family, a central class of serine/threonine kinases in the signal transduction pathway, consists of at least eight isoenzymes [1–3]. This class of protein kinases has received a great deal of attention because they are involved in a variety of cellular processes such as proliferation, differentiation and secretion [4]. PKC isoenzymes are activated by lipid activators such as 1,2-*sn*-diacylglycerol (DAG), which is generated as second messenger by different extracellular signals, as well as by tumour-promoting phorbol esters. Furthermore, the activation process involves an increase in intracellular Ca^{2+} concentrations (at least for the Ca^{2+} -dependent isoenzymes α , β and γ), which leads to a translocation of PKC to the cellular (or nuclear) membrane [1–6]. There, membrane-associated phospholipids, preferentially phosphatidylserine (PS), are thought to act as cofactors for activation [5,6]. Binding of DAG is thought to produce conformational changes within the PKC molecule, resulting in dislocation of the N-terminal pseudo-substrate region from the active site [5–8], which enables PKC to bind and phosphorylate a number of known as well as so-far undefined target proteins [1–4].

The cell- and tissue-specific expression of distinct PKC isoenzymes suggests that individual members of the PKC family might play a role in defined cellular functions [1–4,9,10]. Over-expression of PKC α and β I by introduction of the corresponding cDNAs into fibroblasts has been shown to alter the pattern of gene expression and various growth properties of these cells [11–13], indicating that these two isoenzymes act as signal-transducing protein kinases. It is very likely that PKC isoenzymes play a role in cell transformation and that they are of central

importance in the process of carcinogenesis. However, the specific roles of distinct PKC isoenzymes in different cellular responses and signal transduction pathways await elucidation, and experimental evidence is limited.

In view of this important question, we have carried out a detailed biochemical characterization of the human PKC isoenzymes α and β I, purified from recombinant-baculovirus-infected insect cells, in order to investigate differences with respect to substrate specificity, ATP requirement, activation by trypsin or by Ca^{2+} and a variety of different (phospho)lipids and phosphoinositides. We have focused on the activation of PKC α and β I by $\text{PtdIns}(4,5)\text{P}_2$ compared with DAG as activator and by PtdIns compared with PS as lipid cofactor, since this has been a matter of controversy in recent reports [14–17]. Though no differences in activation were observed between the two isoenzymes studied, our data clearly demonstrate that highly purified PKC α and β I can be fully activated by a combination of Ca^{2+} , $\text{PtdIns}(4,5)\text{P}_2$ and PtdIns . Furthermore, our data indicate that the number of phosphate residues on the respective phosphoinositides determines their potency to act as either activator or cofactor for activation of PKC. These findings may have physiological relevance with respect to the activation of PKC isoenzymes at phosphoinositide-containing intracellular membranes distinct from the plasma membrane, e.g. the endoplasmic reticulum or the nuclear membrane.

EXPERIMENTAL

Cell culture

Sf158 cells were grown at 27 °C in TC-100 medium (J. R. H. Biosciences, Lenexa, KA/Biozol, Eching, Germany)

Abbreviations used: DAG, *sn*-1,2-diacylglycerol; EGF, epidermal growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; PS, phosphatidylserine.

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containing 5% fetal-bovine serum (J. R. H. Biosciences). Procedures for cell culture, baculovirus infection and isolation of viral genomic DNA have been described [18].

Plasmid constructions

The baculovirus expression vector pVL1393 was generously provided by Dr. M. Summers (Texas A & M University, College Station, TX, U.S.A.). The transfer vectors pVL1393-PKC α and pVL1393-PKC β I were constructed as follows: the PKC α *EcoRI* cDNA fragment [19] (generously provided by G. Finkenzeller from our Institute) was ligated into pVL1393 cut with *EcoRI*. The *EcoRI/SmaI* PKC β I cDNA fragment [20] was ligated in pVL1393 cut by *EcoRI* and *SmaI*. The 5' non-coding sequence of the PKC β I cDNA was thereby shortened by 39 base pairs, which facilitated expression in the Sf158 cells. In contrast, the PKC β I cDNA containing the complete 136-base-pair GC-rich 5' non-coding sequence showed no significant expression in Sf158 cells. Standard procedures were carried out as described [18].

Identification of recombinant virus and protein

Production of recombinant baculovirus, infection, immunoblotting and PKC-activity assays were carried out as described in [21], except that the cell-lysis buffer was PBS, 0.02 M EDTA, 0.02 M EGTA and 50 μ g/ml phenylmethanesulphonyl fluoride (PMSF). Anti-(rabbit brain PKC α and PKC β) monoclonal antibodies MC-3a (α) and MC-2a (β) (Seikagaku Kogyo Co., Tokyo, Japan) were used for Western-blot analysis.

Purification of PKC isoenzymes from baculovirus-infected insect cells

Cells were propagated in 1.0-litre Techne stirrers in water-bath vessels (MCS104L and MWB-10L; Techne, Princeton, NJ, U.S.A.) in 0.5 or 1.0 litre of serum-free Ex-Cell 401 medium (J. R. H. Biosciences) to $(2-4) \times 10^6$ cells/ml and infected with 10^9-10^{10} plaque-forming units of recombinant baculovirus. Cells were harvested 72 h post infection, pelleted at 1000 *g* for 15 min, washed once with ice-cold PBS and re-pelleted.

The following operations were carried out at 4 °C. Chromatography columns were connected to an f.p.l.c. system (Pharmacia, Freiburg, Germany). Insect cell pellets (2×10^9 cells) were lysed in buffer A (50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 1 mM PMSF, 10 mM benzamidine, 20 μ g/ml leupeptin) in the presence of 2 mM CaCl_2 and 3 mM MgCl_2 . To determine the amount of kinase activity obtained from the original lysate, a portion of cell lysate was directly adjusted to 5 mM EGTA and 3 mM EDTA. After incubation for 30 min, this sample was centrifuged at 40000 *g* for 45 min. In the resulting supernatant (designated S_{40} EGTA), total PKC activity was measured under standard conditions (see below). The original lysate (without addition of EGTA and EDTA) was centrifuged at 40000 *g* for 15 min and the resulting pellet washed in buffer A containing 0.2 mM CaCl_2 . Subsequently, the pellet was resuspended in buffer A containing 5 mM EGTA and 3 mM EDTA and shaken on ice for 45 min before centrifugation at 200000 *g* for 30 min. The resulting supernatant, greatly enriched in PKC, was named S_{200} Ca^{2+} . This membrane association/dissociation step represents a modification of a method described by Wolf *et al.* [22].

The S_{200} Ca^{2+} supernatant (200 mg of protein/60 ml) was applied to a DEAE-Fractogel column (26 mm $\times$ 30 mm, 15 ml; Merck, Darmstadt, Germany) equilibrated with buffer B (20 mM

Tris/HCl, pH 7.5, 1 mM dithiothreitol, 0.5 mM PMSF, 5 mM benzamidine, 1 mM EDTA, 1 mM EGTA), and PKC activity was eluted with a linear salt gradient (50 ml) of 0–0.4 M NaCl in buffer B (2.5 ml/min; 2.5 ml fractions). The PKC-containing fractions (6 mg of protein/20 ml) were adjusted to 1 M NaCl and then applied to a column of phenyl-Sepharose (16 mm $\times$ 25 mm, 5 ml; Pharmacia) equilibrated in buffer B containing 1 M NaCl. Subsequently, PKC was eluted with a linear descending salt gradient (40 ml) from 1 to 0 M NaCl in buffer B (1.5 ml/min; 3 ml fractions). Fractions containing PKC activity were pooled (1 mg of protein/18 ml), diluted with 4 vol. of buffer B and loaded on to a Mono Q column (5 mm $\times$ 50 mm, 1 ml; Pharmacia) equilibrated in buffer B. PKC activity was eluted with a linear salt gradient (20 ml) of 0–0.5 M NaCl (0.5 ml/min; 1 ml fractions) in buffer B.

PKC-containing fractions were detected by activity assays as described [21], with histone III-S as substrate. Analysis of the PKC-containing fractions was performed on SDS/9%-polyacrylamide gels with subsequent Coomassie Blue staining or by autophosphorylation assays in the mixed-micellar system (see below) with subsequent SDS/PAGE (12% gels) and autoradiography. The enzyme fractions were stored in portions at -70 °C in buffer B containing 40% glycerol, 0.05% Triton X-100 and 0.5 mg/ml soybean trypsin inhibitor without loss of activity.

Characterization of purified PKC isoenzymes

The purified PKC isoenzymes were characterized in the mixed-micellar assay [23] modified to contain 0.03% (instead of 0.3%) Triton X-100 and the various lipids in the concentration range 0.1–100 μ g/ml (concentration in mol% are described in the corresponding Figure legends). The desired amounts of lipids (Sigma, Deisenhofen, Germany, and Boehringer, Mannheim, Germany) were dissolved in chloroform/methanol (19:1, v/v) in the presence of 200 μ M butylated hydroxytoluene. The solvent was then removed under vacuum and the lipids redissolved (as a 10-fold-concentrated stock solution) in 20 mM Tris/HCl (pH 7.5)/0.3% Triton X-100 by vigorous mixing and incubation at 40 °C for 5 min. PKC activity was determined in 40 μ l assays containing 50 mM Tris/HCl, pH 7.5, 1 mM free Ca^{2+} , 2.5 mM free Mg^{2+} , 10 μ g/ml epidermal-growth-factor (EGF) receptor peptide (amino acids 650–658; AUSPEP, Australia), 100 μ M [γ - ^{32}P]ATP (100–200 c.p.m./pmol), 4 μ l of the micellar lipids and 2–10 ng of purified PKC (Mono Q eluate; 0.1 mg of protein/ml). After incubation for 5 min at 30 °C, the reaction was stopped by addition of 1 ml of 5% H_3PO_4 . Subsequently, the peptide was collected on P81-paper discs (Whatman, Maidstone, Kent, U.K.) that were washed extensively with 5% H_3PO_4 . Radioactivity was determined by Čerenkov counting.

For the trypsin-digestion experiments, PKC Mono Q fractions without any protease inhibitor were preincubated at 30 °C in PKC assay buffer without Ca^{2+} and Mg^{2+} containing 1 μ g/ml trypsin (Sigma). The digestion was stopped by the addition of soybean trypsin inhibitor (0.2 mg/ml) and PKC activity was assayed as described above.

RESULTS

Expression and purification of PKC isoenzymes α and β I

Recombinant baculoviruses harbouring the cDNAs of human PKC α and β I were used to infect Sf158 insect cells. To test for expression of the isoenzymes, crude cell extracts were analysed by SDS/PAGE and Western blotting, using isoenzyme-specific anti-PKC antibodies. As shown in Figure 1, only insect cells

Figure 1 Immunoblot analysis of human PKC α and human PKC β I from AcMNPV-PKC α - and AcMNPV-PKC β I-infected insect cells

Sf158 cells were mock-infected (lanes 1, 5 and 9), infected with wild-type baculovirus AcMNPV (lanes 2, 6 and 10), infected with AcMNPV-PKC β I (lanes 3, 7 and 11) or infected with AcMNPV-PKC α (lanes 4, 8 and 12). Cell lysates (20 μ g of protein per lane) were submitted to SDS/PAGE and Western blotting. Blots were incubated with or without anti-PKC antibodies (anti-PKC- α) against the respective PKC isoenzyme: lanes 1–4, anti-PKC α ; lanes 5–8, anti-PKC β ; lanes 9–12, no antibodies. Subsequently, blots were incubated with anti-mouse IgG alkaline phosphatase-conjugated secondary antibodies. The positions of marker proteins are indicated on the left; the arrow marks the position of PKC.

Table 1 Purification of human PKC α and β I from baculovirus-infected insect cells

Details of cell culture, infections and purifications are described in the Experimental section. S₄₀ EGTA and S₂₀₀ Ca²⁺ represent the respective post-microsomal supernatants, and DEAE-Fractogel, Phenyl-Sepharose and Mono Q represent the pooled fractions with PKC activity from the respective chromatography. One unit of PKC activity is defined as the amount of enzyme catalysing the incorporation of 1 nmol of ³²P into histone III-S under standard assay conditions.

Enzyme	Purification step	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification factor (fold)
PKC α	S ₄₀ EGTA	96	35	100	1
	S ₂₀₀ Ca ²⁺	19	63	36	2
	DEAE-Fractogel	4.2	199	20	6
	Phenyl-Sepharose	0.7	779	10	23
	Mono Q	0.15	3006	6	87
PKC β I	S ₄₀ EGTA	174	9	100	1
	S ₂₀₀ Ca ²⁺	26	33	57	4
	DEAE-Fractogel	6	88	34	10
	Phenyl-Sepharose	1	343	21	40
	Mono Q	0.3	597	10	70

infected with the appropriate recombinant baculovirus express the respective PKC isoenzyme. Both PKC α and β I show the expected molecular mass of about 80 kDa, and no antigenic degradation products are visible.

The PKC isoenzymes were purified by a four-step procedure (Table 1). Cells from suspension culture were lysed in the presence of Ca²⁺. Under these conditions, PKC translocates to the membrane fraction, which was harvested by centrifugation. After extensive washing, the membrane pellet was redissolved in a buffer containing EGTA, which leads to dissociation of PKC from the membrane fraction and enrichment in the resulting supernatant (named S₂₀₀ Ca²⁺). In this membrane association/dissociation step, PKC activity was separated from the bulk (> 80%) of other cellular proteins (Table 1). This method,

originally described for erythrocyte vesicles [22], has thus proved to be a powerful tool for the purification of Ca²⁺-dependent PKC isoenzymes from baculovirus-infected insect cells.

The subsequent purification protocol, including chromatography on DEAE-Fractogel, phenyl-Sepharose and Mono Q (Table 1), was carried out at 4 °C within 12 h in order to avoid proteolytic degradation of PKC. No significant differences between the two PKC isoenzymes with respect to the purification procedure were observed. The low purification factor of the Mono Q fraction (Table 1) reflects the high levels of expressed recombinant protein, which represent 2.6% (PKC α) and 1.7% (PKC β I) of the starting material (S₄₀ EGTA) calculated from the final amount of protein (0.15 and 0.3 mg respectively) and the recovery of 6 and 10% respectively (Table 1). The purity of the PKC-containing fractions from the different steps of purification was analysed by SDS/PAGE and Coomassie Blue staining, which demonstrate the substantial homogeneity of the final Mono Q fraction (Figure 2a; only shown for PKC β I). The purity and yields of PKC α and β I in this study are comparable with those described for purifications of recombinant PKC α , β II and γ [24–28] and PKC ϵ [29] by use of affinity chromatography on serine- or threonine-Sepharose.

Both PKC isoenzymes were further analysed by an auto-phosphorylation assay in the absence or presence of PS/DAG (Figure 2b; only shown for PKC β I). With the final Mono Q fraction, only an 80 kDa phosphoprotein is observed whose phosphorylation is strictly PS/DAG-dependent, thus fulfilling two important properties of native PKC.

Substrate specificity and ATP requirement of PKC isoenzymes α and β I

The substrate specificity and ATP requirement of the two human PKC isoenzymes were determined from the appropriate kinetics (results not shown), by using histone III-S and EGF-receptor peptide as substrates [30–32]. The latter contains the Thr-654 residue reported to be the phosphorylation site of PKC within the EGF receptor *in vivo* [30].

For both isoenzymes, K_m values for ATP (about 27 μ M), the EGF receptor peptide (about 3 μ g/ml) and histone III-S (about 33 μ g/ml) are quite similar. However, K_m values differ significantly between histone III-S and the EGF receptor peptide. A comparison of our data with the findings of Marais et al. [31] indicates that the difference between histone III-S and the EGF receptor peptide does not necessarily reflect a difference of peptide versus holoprotein substrate. In their study, carried out with PKC α , β I and γ purified from calf brain, the K_m values for several peptide substrates (amongst them the EGF receptor peptide) differed significantly [31]. Recently, it has been shown that PKC ϵ and δ exhibit quite different K_m values for the EGF receptor peptide compared with the Ca²⁺-dependent isoenzymes [32]. Though the substrates used in the present study did not reveal any differences in the specificity of the PKC isoenzymes investigated, further experimental work using known PKC substrates as holoproteins and protein-derived peptides may provide us with more accurate data and conclusions.

Activation of PKC isoenzymes α and β I by tryptic digestion

After translocation to the cellular membrane, PKC is cleaved at a hinge site between the regulatory and the catalytic domain by the Ca²⁺-dependent protease calpain [1–3]. This leads to the removal of the regulatory subunit (and the inherent pseudo-substrate region) and thus results in a constitutively active phospholipid-independent catalytic subunit of PKC. This pro-

Figure 2 SDS/PAGE (a) and phosphorylation pattern (b) of PKC β I from baculovirus-infected insect cells at different purification steps

Analysis of the different purification steps by SDS/PAGE and subsequent Coomassie Blue staining or by phosphorylation assays was performed with the following fractions (see the Experimental section): S₄₀ EGTA (cell extract); S₂₀₀ Ca²⁺ (membrane-derived supernatant); DEAE (DEAE-Fractogel pool); Phenyl (phenyl-Sepharose pool); Mono Q (Mono Q pool). For SDS/PAGE analysis, the indicated amounts of protein were loaded. For the phosphorylation analysis, equal volumes (2 μ l) of each fraction were incubated in the mixed-micellar assay as described in the Experimental section, in the absence of substrate and in the absence or presence of activating lipids (22 mol% PS; 2.6 mol% DAG) as indicated. The autoradiograph is shown and the positions of marker proteins on the stained gel are indicated. The position of PKC is marked by an arrow.

teolytic fragment is highly susceptible to further degradation and inactivation, a process assumed to be the basis of the phorbol-ester-induced down-regulation of PKC [1–3].

The physiological role of this proteolytic cleavage is still not fully understood. It is not known (i) whether or not the phospholipid-independent catalytic fragment represents a membrane-independent, even cytoplasmic or nuclear, form of activated PKC, and (ii) whether the proteolytic cleavage by calpain requires a previous change in PKC conformation induced by phospholipid binding. Thus one could hypothesize that an

Figure 3 Activation by trypsin of purified human PKC α and β I

Purified PKC α (10 μ g of protein; $\circ$, $\square$) and PKC β I (3.6 μ g of protein; $\bullet$, $\blacksquare$) were incubated in a final volume of 100 μ l with 1 μ g/ml trypsin for 15 min at 30 $^{\circ}$ C. At the time points indicated, 20 μ l samples were taken and PKC activity was assayed in the mixed-micellar system in the absence ($\square$, $\blacksquare$) or presence ($\circ$, $\bullet$) of PS/DAG (22 and 2.6 mol% respectively). For details see the Experimental section. In this experiment (one of two independent experiments), 100% PKC activity represented 22 000 c.p.m. (PKC α) and 15 000 c.p.m. (PKC β I) respectively.

increase in intracellular Ca²⁺ concentrations alone, causing translocation of PKC to the membrane and activation of calpain, is sufficient for activation of PKC, though maybe only transiently.

In order to investigate these questions, we have examined the activation of purified PKC α and β I by trypsin which exerts the same proteolytic cleavage as calpain. The appearance of both proteolytic fragments was monitored by SDS/PAGE and Coomassie Blue staining (results not shown). PKC β I is rapidly activated by trypsin reaching the activity observed in the presence of PS/DAG within 5 min (Figure 3). Then activity slightly declines, probably due to further proteolysis. In contrast, PKC α appears to be rather insensitive to trypsin treatment; its activity slowly increases to only 50% of the activity obtained in the presence of PS/DAG and then remains unaltered during the subsequent 10 min of the experiment (Figure 3). It is most unlikely that this finding is due to a balance between further proteolytic activation and degradation. Instead, it indicates that PKC α as such is rather insensitive to proteolytic activation and degradation, and thus clearly differs from PKC β I. From these findings, one could conclude that, for PKC β I, an increase in intracellular Ca²⁺ concentrations alone might be sufficient for translocation and subsequent proteolytic activation. Our data are in agreement with the report by Huang et al. [33] that, in rat basophilic leukaemia cells, PKC β seems to be much more susceptible to phorbol 12-myristate 13-acetate (PMA)-mediated down-regulation than PKC α . Nevertheless, any conclusions on the relative stability of PKC α and β I *in vivo* should be drawn with care, since recently Van den Berghe et al. [34] reported a PMA-mediated down-regulation of PKC α , but not of PKC β I, in a human colonic cell line.

Activation of PKC α and β I by Ca²⁺, PS, DAG and PtdIns(4,5)P₂

The characterization of the purified PKC isoenzymes with respect to different activators was performed in a mixed-micellar assay [23] which is reported to minimize Ca²⁺-derived artifacts (by vesicle fusion) and to increase largely the specificity of phospholipid action [23]. In order to maintain a constant hydrophobic environment, variable phospholipid or phosphoinositide concentrations were prepared within Triton X-100 micelles at a constant Triton concentration of 0.03% (see the Experimental section). Though this Triton concentration is one-tenth of that used by Bell and co-workers [5,17,23] and close to the critical micellar

Figure 4 Ca^{2+} requirement of PKC α in the absence or presence of lipid activators

PKC activity was determined in mixed-micellar assays with increasing concentrations of free Ca^{2+} in the presence of 12 mol% PS alone (○) or combined with either 3 mol% DAG (■) or 0.85 mol% $\text{PtdIns}(4,5)\text{P}_2$ (●). In this experiment (one out of five independent experiments), 100% PKC activity represented 6500 c.p.m.

concentration of 0.017%, it allowed us to decrease the amounts of phospholipid likewise without changing the actual micelle composition (if calculated as mol% concentrations). Experiments with varying PS and DAG concentrations, respectively, carried out at constant mol% concentrations in mixed micelles generated by Triton X-100 in the concentration range 0.03–0.3% did not reveal any significant difference in PKC activation (results not shown).

In a first series of experiments, the concentration of free Ca^{2+} was varied within the range 0.1 mM–1 nM, in the presence of either PS alone or of PS/DAG or PS/ $\text{PtdIns}(4,5)\text{P}_2$. The two

Table 2 PKC α activity in the presence of various (phospho)lipid activators and cofactors

PKC activity was determined in the mixed-micellar assay in the presence of 1 μM free Ca^{2+} . The different (phospho)lipids were tested as activators in the absence or presence of 12 mol% (66 μM) PS (columns — PS and + PS respectively), and as cofactors of activation in the absence or presence of 3 mol% (16 μM) DAG (columns — DAG and + DAG respectively). The results of three independent experiments were normalized such that PKC activity in the presence of PS/DAG (12 and 3 mol% respectively) represented 100% activity. Relative PKC activity in the absence of any lipids was 4%.

Lipid	As activator			As cofactor		
	Concn. (mol%)	Relative PKC activity (%)		Concn. (mol%)	Relative PKC activity (%)	
		— PS	+ PS		— DAG	+ DAG
DAG	3	8	100	3	—	8
PS	12	—	36	12	36	100
Phosphatidylcholine	21	6	40	21	6	8
Phosphatidylglycerol	21	8	39	21	8	37
PtdIns	2	6	43	19	6	83
PtdIns4 <i>P</i>	2	4	57	9.5	4	71
PtdIns(4,5) <i>P</i> ₂	1.9	6	94	9	7	16
Cardiolipin	12	27	72	12	26	46

human PKC isoenzymes did not exhibit any differences in Ca^{2+} and activator requirement (results not shown). The results obtained for PKC α are shown in Figure 4. Below a concentration of 50 nM free Ca^{2+} , no activation was observed. At 100 nM free

Figure 5 Activation of PKC α by DAG (a and c) or $\text{PtdIns}(4,5)\text{P}_2$ (b and d) combined with PS or PtdIns

PKC activity was determined in the mixed-micellar assay (number of experiments: three) in the presence of 1 μM free Ca^{2+} . In the presence of increasing concentrations of PS (a and b) or PtdIns (c and d) the following concentrations of DAG were assayed: ○, 0 mol%; ●, 0.3 mol% (1.6 μM); ■, 1.5 mol% (8 μM); ▲, 3 mol% (16 μM). $\text{PtdIns}(4,5)\text{P}_2$ (b and d) was tested in the following concentrations: ○, 0 mol%; ●, 0.17 mol% (0.9 μM); ■, 0.85 mol% (4.5 μM); ▲, 1.9 mol% (9 μM). One mol% of PS corresponds to 5.5 μM , and one mol% of PtdIns to 5.3 μM .

Ca^{2+} and above, the combination of PS/DAG as well as of PS/PtdIns(4,5) P_2 strongly activates PKC, whereas PS alone leads to a partial activation only at rather high concentrations of free Ca^{2+} , i.e. at 10 μM and above. This is in agreement with previous observations that lipid activators reduce the Ca^{2+} concentration required for activation [5,17].

Activation of PKC α and βI by different phospholipids and phosphoinositides

The plasma membrane, which is presumably the major site of PKC activation, is composed of a complex mixture of glycerol- and phospho-lipids that is modulated in the course of signal transduction [35]. Thus a number of different phospholipids and phosphoinositides, besides DAG and PS, may function as physiological activators of PKC and/or cofactors of activation [5,17], and may even do so in an isoenzyme-specific way.

Therefore we have tested several phospholipids and phosphoinositides for their capability to substitute either for DAG as activator or for PS as cofactor of activation for PKC α and βI . Since no difference between the two isoenzymes was observed, only the data obtained with PKC α are shown (Table 2). In the presence of 12 mol% PS and 1 μM free Ca^{2+} , phosphatidylcholine, phosphatidylglycerol and PtdIns fail to activate PKC, whereas cardiolipin, PtdIns4P and PtdIns(4,5) P_2 are intermediate or potent activators (Table 2). In contrast, PtdIns and PtdIns4P are rather potent cofactors of activation (in the presence of 3 mol% DAG and 1 μM free Ca^{2+}), whereas cardiolipin and phosphatidylglycerol are intermediate cofactors, and phosphatidylcholine and PtdIns(4,5) P_2 fail to show any effect (Table 2). As to the activating potential of phosphoinositides, the data summarized in Table 2 suggest that an increasing number of phosphate groups within the phosphoinositides enables them to act as activators of PKC, at the same time diminishing their capability to act as cofactors of activation.

Activation of PKC α and βI by PtdIns and PtdIns(4,5) P_2

Since PtdIns(4,5) P_2 and PtdIns appeared to be capable of substituting for DAG and PS as activator and cofactor of activation respectively, the effects of PS, PtdIns, DAG and PtdIns(4,5) P_2 , in the presence of 1 μM Ca^{2+} , were investigated in more detail (Figure 5; shown for PKC α). With increasing concentration of PS (which as such has only a slight effect on PKC activation), both DAG and PtdIns(4,5) P_2 are potent activators of PKC (Figures 5a and 5b). However, both of them require a minimal concentration of PS. Whereas activation by DAG is only observed in the presence of 12 mol% PS, PtdIns(4,5) P_2 activates PKC to the same extent already at PS concentrations around 6.5 mol%. As Figures 5(c) and 5(d) show, PtdIns can substitute for PS as cofactor of activation, though clearly higher concentrations of PtdIns are required to obtain full activation by DAG or PtdIns(4,5) P_2 . Compared with DAG, however, PtdIns(4,5) P_2 seems to be an equally or even more potent activator of PKC in the presence of either cofactor (Figures 5b and 5d).

DISCUSSION

The existence of, to date, eight PKC isoenzymes that exhibit remarkable differences in their structure, in their expression pattern in different tissues and in their mode of activation [1–4]

has prompted the hypothesis that PKC isoenzymes represent mediators in quite distinct signal-transduction pathways. In order to substantiate this hypothesis, it is necessary to analyse homogeneous preparations of PKC isoenzymes with respect to their biochemical properties under conditions *in vitro* which mimic or come quite close to the situation presumed to be present in the intact cell.

In the present study, we have taken advantage of the possibility to express and purify the human PKC isoenzymes α and βI from recombinant-baculovirus-infected insect cells. These distinct and homogeneous fractions (Figures 1 and 2, Table 1) permitted an evaluation with respect to their susceptibility to proteolytic activation (Figure 3), to their Ca^{2+} -dependence (Figure 4) and to the mode of activation by various phospholipid activators (Table 2 and Figure 5).

The variety of components of inositol lipid metabolism [35] generated and involved in cell signalling has raised the question whether or not PKC isoenzymes may be activated by (phospho)lipids distinct from DAG and PS. Convincing evidence has been accumulated that, besides an increase in the cytosolic Ca^{2+} concentration (for the classical PKC isoenzymes), actually two components are required for full activation of PKC [5,6]: (i) PS as a cofactor of activation, already present in the membrane, and (ii) DAG as the actual activator that is produced by the signal-mediated breakdown of membrane-associated PtdIns(4,5) P_2 and maybe other phosphoacylglycerols. Recently, Chauhan et al. [14–16] have demonstrated that PtdIns(4,5) P_2 can substitute for DAG as activator by interacting directly with the DAG/phorbol-ester-binding site of PKC. This finding has been supported by an intensive study carried out by Lee and Bell [17], though some controversy remains with respect to the binding site of PtdIns(4,5) P_2 [14,16,17]. However, in all cases activation of PKC by PtdIns(4,5) P_2 did not reach the extent observed with DAG, which might be due to the fact that both groups used a purified PKC preparation from rat brain that may contain a portion of PtdIns(4,5) P_2 -insensitive PKC isoenzymes. The reason for rather low levels of activation by PtdIns(4,5) P_2 for purified recombinant PKC isoenzymes α , βII and γ [17] is unknown. An activation of PKC by PtdIns instead of PS has been reported for a mixture of conventional PKC purified from rabbit and rat brain [32,36].

Using highly purified PKC isoenzymes α and βI , we could demonstrate a full activation by PtdIns(4,5) P_2 at rather low concentrations of Ca^{2+} and PS (Figure 4). This indicates that PtdIns(4,5) P_2 , like DAG, is able to increase the affinity of these isoenzymes to Ca^{2+} . The obvious sensitivity of the system to phosphoinositides as potential activators of PKC prompted us to extend our investigation to various phospholipids and phosphoinositides as either activators of PKC or cofactors of activation (Table 2). Besides cardiolipin, which seemed to exert an intermediate effect as both activator and cofactor, the phosphoinositides exhibit a striking capacity to act as potent activators or cofactors of activation that is reciprocally dependent on the number of phosphate groups present. PtdIns is most potent in substituting for PS as a cofactor of activation, whereas PtdIns(4,5) P_2 is as effective as DAG in activating the respective PKC isoenzyme. The latter is rather astonishing, since PtdIns(4,5) P_2 and DAG differ considerably in their structure and polarity. From this point of view, an investigation of the activating potential of phosphoinositides phosphorylated in the 3' position would be interesting and could reveal the role of the respective phosphatidylinositol kinase(s).

Though no isoenzyme-specific differences of activator/cofactor requirement between PKC α and βI could be observed, there may be differences from other PKC isoenzymes. Recently,

cardiolipin has been described to be a potent activator of PKC ϵ [37], though it seems at best an intermediate activator for PKC α and β I (Table 2). Our findings indicate the possibility that (at least) these two isoenzymes can be activated by intermediates of inositol metabolism, i.e. PtdIns and PtdIns(4,5) P_2 . This implies that PKC activity may depend not necessarily on the production of DAG by phospholipase C, but also on the activity of the phosphatidylinositol kinases. Moreover, one could postulate that a Ca^{2+} -dependent translocation of these PKC isoenzymes to membranes distinct from the plasma membrane, e.g. the endoplasmic reticulum or the nuclear membrane, could occur, and thus may lead to activation by the inherent phosphoinositides of those cellular compartments. It should be noted that all cellular membranes (plasma, Golgi, nuclear and microsomal) seem to exhibit a rather similar content of PS and PtdIns, though the amount of PtdIns actually exceeds the amount of PS [38]. For several PKC isoenzymes, among them PKC α and β II, a translocation to the nuclear membrane has been reported [10,39]. It has been postulated [15] that the binding of PtdIns(4,5) P_2 or DAG to the regulatory domain of PKC may influence the substrate specificity of the catalytic domain, and indeed an inter-domain influence on substrate specificity has been demonstrated for PKC ϵ recently [40,41]. Further investigations will be necessary to show whether or not different activators can modify the substrate specificity of the conventional PKC isoenzymes.

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