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Phospholipase D activation in fibroblast membranes by the α and β isoforms of protein kinase C

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Abstract

The regulation of phosphatidylcholine-hydrolyzing phospholipase D (PLD) by protein kinase C (PRC) in membranes of Chinese hamster lung fibroblasts (CCL39) was studied using conventional PKC isoforms α, β and γ isolated from rat brain and recombinant PKC isoforms. Cells were incubated with [^{14}C]choline to label endogenous phosphatidylcholine before membranes were prepared and assayed for release of [^{14}C]choline. PKCα was the most potent activator of PLD, producing a maximal effect at approximately 0.1 μg/ml. PKCβ also stimulated PLD but was less potent and less efficacious, whereas PKCγ was ineffective. Stimulation required addition of a PKC activator, but the isoform specificity was the same whether phorbol 12-myristate 13-acetate (PMA) or Ca^{2+} was used. Recombinant Ca^{2+}-independent PKC isoforms δ, ε, and ζ failed to stimulate PLD, but recombinant PKCβ_1 stimulated PLD in a manner similar to the purified brain PKCβ. Immunoblot analysis of the soluble fraction of CCL 39 fibroblasts detected only the α and ζ isoforms of PKC. The results suggest that PKCα and β are activators of PLD and that PKCα is responsible for the activation in these fibroblasts.

Key words: Phospholipase D, Protein kinase C, Phosphatidylcholine, Calcium

1. Introduction

Activation of phosphatidylcholine-hydrolyzing phospholipase D (PLD) occurs in response to hormones and growth factors in many cell types [1]. Regulation of the enzyme may occur by mechanisms involving G-proteins [2–4], Ca^{2+} [4,5], unsaturated fatty acids [6], and PKC [7–16]. Much attention has focused on the role of PKC in the regulation of PLD. Several studies indicate that down-regulation of PKC by long-term exposure to phorbol ester abolishes the ability of agonists to acutely stimulate PLD [7–9]. In addition, kinase inhibitors frequently [8–11], but not always [12–15], block activation of PLD by phorbol ester and other agonists, suggesting the involvement of both PKC-dependent and PKC-independent mechanisms. Recently, we obtained direct evidence that PKC activates a PLD present in membranes from Chinese hamster lung (CCL39) fibroblasts [16]. Surprisingly, the stimulation apparently did not require ATP-dependent phosphorylation, suggesting that PKC may interact directly with PLD and activate it by an allosteric mechanism.

PKC is a family of at least ten isoforms that are differentially regulated by Ca^{2+} and by phorbol esters, which substitute for the physiological regulator diacylglycerol [17,18]. Two regions of conserved sequence in the PKC regulatory domain which constitute phorbol ester and Ca^{2+} binding sites have been identified. The conventional PKC isoforms (cPKC) α, β_1, β_2 and γ contain both conserved regions, whereas novel PKC isoforms (nPKC) such as δ and ε lack the Ca^{2+}-binding region [17,18]. Atypical PKC isoforms (aPKC), which include PKCζ, lack the Ca^{2+}-binding region and contain only half of the phorbol ester binding region [17,18], and are insensitive to both activators [19].

Although much is known about the regulation of PKC, the role of the various isoforms in regulation of cellular processes is poorly understood. In this study, we have examined which isoforms are involved in regulation of PLD in membranes prepared from CCL39 fibroblasts.

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Abbreviations: PLD, phospholipase D; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2. Materials and methods

2.1. Determination of PLD activity in membranes

Chinese hamster lung fibroblasts (CCL39) were grown, prelabelled with [^{14}C]choline, and homogenized for membrane preparation as described previously [16]. PLD activity was assayed by measuring the production of [^{14}C]choline. Incubations contained 20 mM HEPES pH

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7.4, 1.1 mM EDTA, 2.1 mM EGTA, 3 mM $MgCl_2$, 5 mg/ml bovine serum albumin, and 0.1–0.2 mg/ml membrane protein in a total volume of 0.1 ml and were run at 37°C for 30 min. In some cases, $CaCl_2$ was added and the free Ca^{2+} concentration was calculated using the COMICS program. Reactions were terminated with 2.5 ml methanol/chloroform/water (100:50:40) and the phases were separated by addition of 0.5 ml chloroform and 0.5 ml water. The upper phase was mixed with 2 ml of water and centrifuged to produce an aqueous phase from which choline was separated from other water-soluble choline metabolites by ion-pair extraction using sodium tetraphenylboron [20]. A 3 ml aliquot of the upper phase was mixed with 1.5 ml of 10 mg/ml tetraphenylboron in 3-heptanone. After centrifugation, 1 ml of the upper (heptanone) phase was subjected to scintillation counting. This procedure gave identical results to those obtained when choline metabolites were separated by thin layer chromatography.

2.2. Purification of protein kinase C isoforms

PKCα, β and γ were purified from rat brain as described previously [21]. After resolution by hydroxylapatite chromatography, the identity of the isoforms was confirmed by immunoblot analysis using specific antisera. Each of the isoforms gave a single band of apparent molecular weight 80 kDa after SDS-PAGE and silver staining. Recombinant human PKC isoforms were expressed using a baculovirus-Sf9 cell expression system and partially purified by DEAE-Sephacel chromatography [22]. PKC activity was determined using a reaction mixture (0.1 ml) containing 25 mM Tris pH 7.5, 0.2 mg/ml myelin basic protein, 5 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (5×10^5 cpm/nmol), 1 mM dithiothreitol, and 0.1 mg/ml leupeptin, and the assay conditions described previously [23].

2.3. Immunoblot analysis of PKC isoforms in CCL39 cells

Homogenates prepared from rat brain [21] and CCL39 cells [16] were centrifuged at $100{,}000 \times g$ for 30 min. Samples of the soluble fraction (4 μg protein) were subjected to 10% SDS-PAGE. The gels were equilibrated with 48 mM Tris, 39 mM glycine, 1.3 mM SDS, and 20% methanol, and proteins were transferred to Immobilon-P membranes using a semi-dry transfer apparatus (Bio-Rad) for 1 h at 15 V. The membranes were blocked with 1% bovine serum albumin, 1 ml/100 ml goat serum, 20 mM Tris pH 7.5, 0.9% NaCl, and 0.1% Tween 20 for 30 min. They were then incubated with 1 μg/ml specific anti-PKC antisera and, in some cases, 1 μg/ml PKC isozyme-specific peptide in the same solution. Following incubation with biotinylated anti-rabbit IgG (5 μg/ml) in the same solution without albumin or serum, the blots were developed with a Vectastain alkaline phosphatase ABC kit.

2.4. Materials

[^{14}C]Choline and [γ-^{32}P]ATP were from DuPont-New England Nuclear. Myelin basic protein, PMA, fetal bovine serum, 3-heptanone, and sodium tetraphenylboron were from Sigma. Phosphatidylserine was from Avanti. Immobolin-P membranes were from Millipore. Biotinylated anti-rabbit IgG and the Vectastain alkaline phosphatase ABC kit were from Vector Laboratories. Precast polyacrylamide gels were from Novex. Dulbecco's modified Eagle's Medium and polyclonal antibodies recognizing PKC α, β, γ, ε and ζ were from Gibco/BRL. Anti-PKCδ was from Research and Diagnostic Antibodies. Ready Organic scintillation cocktail was from Beckman.

3. Results and discussion

We found previously that purified rat brain PKC stimulated PLD in CCL39 membranes if PMA was also present [16]. Fig. 1 shows that Ca^{2+}, another PKC activator, stimulates PLD, as measured by [^{14}C]choline release, in the presence of PKC. Maximal stimulation occurred at 200 nM Ca^{2+}, a concentration lower than that required for stimulation of PKC activity in vitro [23]. It is possible that the lower Ca^{2+} requirement for PLD activation observed here is due to the presence of endogenous PKC activators, such as diacylglycerol. Relatively high Ca^{2+}

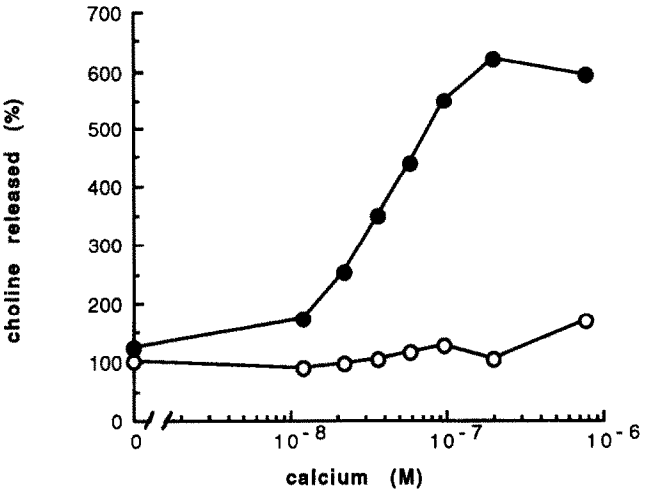

Fig. 1. Ca^{2+} dependence of PLD activation by PKC. [^{14}C]Choline production from fibroblast membranes was determined in the absence (○) or presence (●) of 0.3 μg/ml rat brain PKCα. Assay conditions were as described in section 2. All incubations contained 2.1 mM EGTA and $CaCl_2$ was added to obtain the indicated free Ca^{2+} concentration. The incubation with '0' calcium contained only EGTA. Values are expressed as the percentage of [^{14}C]choline released in the absence of PKC and added $CaCl_2$.

concentrations stimulate PKC activity in the presence of anionic phospholipids, but diacylglycerol reduces the concentration of Ca^{2+} required for activation [23]. Although some investigations have implicated Ca^{2+} in PLD regulation [4,5], our finding that Ca^{2+} is ineffective in the absence of added PKC indicates that Ca^{2+} does not directly activate PLD in CCL 39 membranes. In platelets, the increase in Ca^{2+} elicited by thrombin has been reported to stimulate PLD independently of PKC, but the mechanism of the Ca^{2+} effect was not identified [5]. In CCL 39 fibroblasts, however, stimulation of PLD by thrombin is completely blocked by protein kinase C down-regulation [7], suggesting that, in this cell type, the increase in cytosolic Ca^{2+} induced by this agonist [24] does not provoke PLD activation independently of PKC.

The rat brain PKC that was found to be capable of activating PLD consisted of a mixture of the α, β and γ isoforms [16]. In order to determine which isoforms were active, they were resolved by hydroxylapatite chromatography [21]. Fig. 2 shows the effect of the isoforms on PLD activity in fibroblast membranes. PKCα was the most potent and efficacious activator, having a maximal effect at approximately 0.1 μg/ml. PKCβ also stimulated PLD with a maximal effect at about 0.4 μg/ml, while PKCγ was ineffective. This pattern of stimulation held whether PMA (Fig. 2B) or Ca^{2+} (Fig. 2C) was used as the activator. To determine if nonclassical PKC isoforms could activate PLD, we made use of recombinant enzymes that had been expressed in Sf9 cells and partially purified by DEAE-Sephacel chromatography [22]. Fig. 3 shows that PKCδ, ε and ζ had no effect on PLD

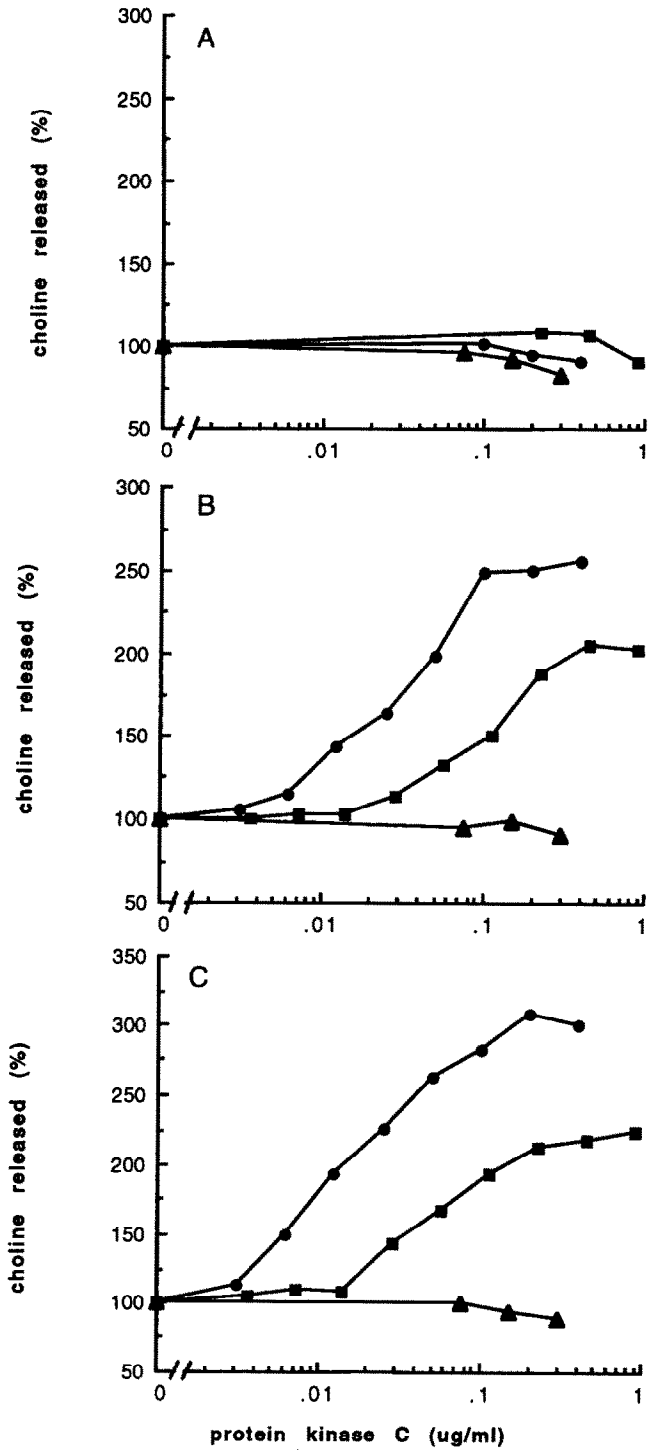

Fig. 2. Effect of purified PKC isoforms on PLD activity. Production of [^{14}C]choline from fibroblast membranes was determined with increasing amounts of PKCα (●), PKCβ (■), or PKCγ (▲) purified from rat brain. The incubations contained A, no activator; B, 0.5 μM PMA; or C, 0.2 μM free Ca^{2+}. Values are expressed in each case as the percentage of [^{14}C]choline produced in the absence of PKC.

activity, while PKCβ_1 activated the enzyme in a PMA-dependent manner. PKCβ_2, which is derived from alternative splicing of the same gene transcript as PKCβ_1 [25], and PKCα also produced a stimulation of PLD (data not shown). The δ, ε and ζ isoforms were shown to be active by their ability to phosphorylate myelin basic protein (their units of activity are given in the legend to Fig. 3).

Immunoblot analysis of the CCL 39 soluble fraction was performed in order to determine which PKC isoforms were present in this cell type. We have shown previously that this soluble fraction contains a PKC responsible for PLD activation in these fibroblasts [16]. Fig. 4 (lane 3) shows that only PKCα and ζ immunoreactivity could be detected. As a control to insure proper detection of the signal for each isoform, a sample of rat brain soluble fraction was included (lane 1). Due to the large amount of nonspecific binding, the identity of the PKC bands was confirmed by blocking with the isoform-specific peptides that were used for raising the antisera (lanes 2 and 4). The anti-PKCζ antisera has been found to recognize not only this isoform, but also PKCα, which migrates at a slightly higher molecular weight [26].

The results show that PKCα and β can activate PLD and suggest that PKCα is the isoform responsible for the stimulation in CCL 39 cells. As activation of PLD by phorbol esters occurs in nearly all cell types [1], it would be expected that the PKC isoforms involved be ubiquitously expressed. Indeed, Westal et al. [27] found PKCα immunoreactivity in all eight rat tissues they examined, while PKCβ_1 and β_2 were present in nearly all tissues. In addition, either PKCα or β is apparently expressed in all cultured cell lines [28].

The finding that PKCβ activated PLD is supported by

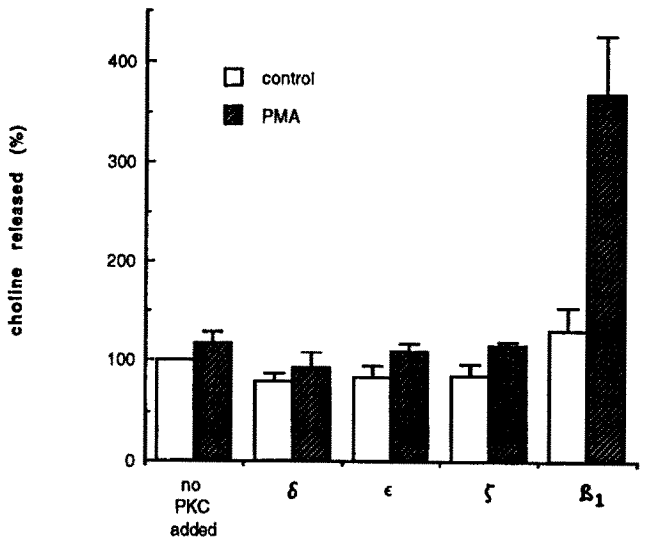

Fig. 3. Lack of PLD activation by recombinant Ca^{2+}-independent PKC isoforms and stimulation by recombinant PKCβ_1. [^{14}C]Choline release from fibroblasts membranes was determined without added PKC or with 2.2 units/ml PKCδ, 1.7 units/ml PKCε, 0.5 units/ml PKCζ, or 0.7 units/ml PKCβ_1. Incubations were run in the absence or presence of 0.5 μM PMA. Values are expressed as the percentage of [^{14}C]choline released in the absence of PKC or PMA. The mean + S.D. of samples run in triplicate is shown.

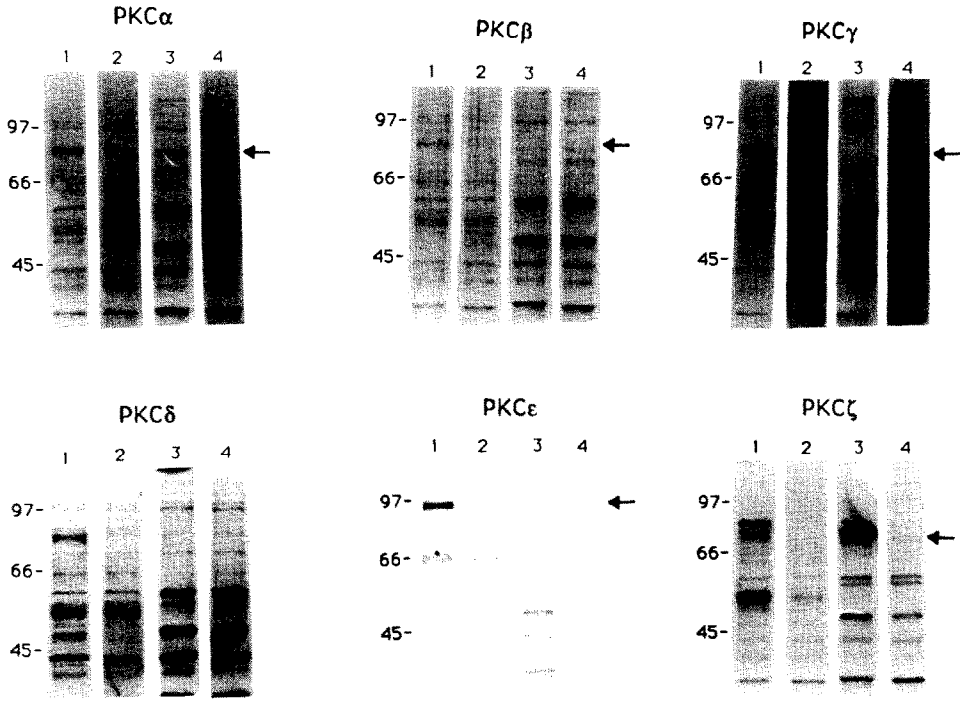

Fig. 4. Immunoblot analysis of PKC isoforms in CCL 39 soluble fraction. Samples (4 μg protein) of rat brain (lanes 1 and 2) or fibroblast (lanes 3 and 4) soluble fractions were subjected to SDS-PAGE and immunoblotted as described in section 2. To demonstrate specific binding, blots were incubated with the appropriate antisera in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of PKC isoform-specific peptide. Arrows indicate the position of the various PKC bands. The position of molecular weight markers is given in kDa.

the demonstration that PKCβ_1 overexpression in rat fibroblasts enhances PMA-stimulated PLD activity [29]. However, recent studies with Swiss 3T3 fibroblasts overexpressing PKCα suggest that this isoform is not involved in the acute stimulation of PLD by PMA, but rather that it plays a role in expression of the PLD enzyme [30]. Also in apparent contradiction to the present results is the recent report suggesting that PKCε is an activator of PLD in mesangial cells [31]. The reasons for the differences between those studies and our results is unknown but may involve the presence of different PLD isoforms in the various cell types. The existence of PLD isoforms has been suggested based on the observation that different preparations of the enzyme differ in substrate, detergent, and cation requirements [32]. It is also likely that PLD activation by PKC takes place by both phosphorylation-dependent and -independent mechanisms. We have obtained evidence that, in CCL 39 membranes, the stimulation of PLD occurs independently of protein phosphorylation [16], raising the possibility that PKC may bind directly to PLD and activate it by an allosteric mechanism. However, experiments using other cell types suggest a dependence on ATP [8–11,33]. Only after PLD has been isolated can the mechanism(s) of activation be tested directly. If, in fact, different PLD isoforms exist, their isolation should allow examination of the possibility that they are differentially regulated by PKC isoforms.

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