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Role of phospholipase D in the activation of protein kinase D by lysophosphatidic acid

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Abstract

Protein kinase D was auto-phosphorylated at Ser916 and trans-phosphorylated at Ser744/Ser748 in Rat-2 fibroblasts treated with lysophosphatidic acid. Both phosphorylations were inhibited by 1-butanol, which blocks phosphatidic acid formation by phospholipase D. The phosphorylations were also reduced in Rat-2 clones with decreased phospholipase D activity. Platelet-derived growth factor-induced protein kinase D phosphorylation showed a similar requirement for phospholipase D, but that induced by 4β-phorbol 12 myristate 13-acetate did not. Propranolol an inhibitor of diacylglycerol formation from phosphatidic acid blocked the phosphorylation of protein kinase D, whereas dioctanoylglycerol induced it. The temporal pattern of auto-phosphorylation of protein kinase D closely resembled that of phospholipase D activation and preceded the trans-phosphorylation by protein kinase C. These results suggest that protein kinase D is activated by lysophosphatidic acid through sequential phosphorylation and that diacylglycerol produced by PLD via phosphatidic acid is required for the autophosphorylation that occurs prior to protein kinase C-mediated phosphorylation.

Keywords: Phospholipase D; Protein kinase D; Phosphorylation; Lysophosphatidic acid; Diacylglycerol

Protein kinase D (PKD, human protein kinase Cμ) is a novel serine/threonine protein kinase which is closely related to the protein kinase C (PKC) superfamily [1]. This lipid-activated kinase shares significant homology to PKC, with amino-terminal cysteine-rich and carboxy-terminal kinase domains. The binding of diacylglycerol (DAG) to PKD [2] also resembles that of most isozymes of PKC. However, PKD is distinguished from PKC because of several characteristics; the presence of a pleckstrin-homology (PH) domain within the regulatory region of PKD, the unique substrate specificity, the insensitivity to PKC inhibitors, and the absence of an autoinhibitory pseudosubstrate domain [1]. PKD is thought to be regulated by PKC through phosphorylation at Ser744/Ser748 in the activation loop [3]. In addition to this trans-phosphorylation site, PKD is auto-phosphorylated at Ser916 upon activation [4] and this phosphorylation was suggested to be important for recruitment of PKD to the Golgi compartment [5].

Recent studies on PKD revealed that it is activated by sequential activation of phospholipase C (PLC) and PKC upon extracellular stimulation [3,6,7]. The βγ subunit complex of heteromeric G-proteins was also suggested as an activating signal for PKD, especially for the Golgi organization [8]. A more recent report has shown that DAG plays an essential role for PKD localization in the Golgi [2]. DAG can also activate PKC but the DAG effect on PKD recruitment to the Golgi seems to be more direct, with interaction with the first cysteine-rich domain of PKD [9]. Interestingly, PKD recruitment to the Golgi was inhibited by propranolol which blocks production of DAG from phosphatidic acid (PA) [2].

Phospholipase D (PLD) [10] is a ubiquitously expressed enzyme which increases cellular PA levels by hydrolysis of phosphatidylcholine. Two mammalian isozymes of PLD (PLD1 and PLD2) have been identified, and they are differently regulated depending on

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upstream signals, including PKC and the small G-proteins Rho and Arf [10]. PKC is a common activator of both PLD1 and PLD2 in vivo although PLD2 shows higher basal activity [11]. However, Rho and its upstream regulator G_{13} activate only PLD1 [11]. Lysophosphatidic acid (LPA), a major bioactive lipid in serum, activates many signaling pathways, including those involving Rho and PLC, through specific receptors coupled to different G protein pathways [12–14]. Since both PLD and PKD are activated by LPA through Rho- and/or PKC-dependent mechanisms [10,15,16], and DAG is required for PKD function, PA produced by PLD might be a possible donor of DAG for PKD activation. In fact, Golgi structure and function are disturbed by the inhibition of PA synthesis by PLD [17]. In the present study, we have examined the dependency of PKD activation on PLD and suggest that PLD can be a key molecule that links Rho/PKC signaling to DAG for PKD activation.

Methods

Reagents. Antibodies specific to PKD, phospho-Ser916, and phospho-Ser744/748 of PKD were purchased from Cell Signaling Technology. LPA was obtained from Avanti Polar Lipids and PDGF was from Invitrogen. 1-butanol, *t*-butanol, and PMA were purchased from Sigma and propranolol was from Calbiochem. G418 was from Invitrogen and bicinchoninic (BCA) protein assay kit was from Pierce.

Cell culture. Rat-2 embryonic fibroblasts were purchased from the American Type Culture Collection and cultured in DMEM containing 10% FBS. Rat-2 mutant clones reduced in PLD activity, Rat2V25 and Rat2V29 [18] were maintained in culture medium containing 0.5 mg/ml G418. For serum-starvation, cells were incubated in DMEM for 24 h without G418.

Western blot analysis. Serum starved Rat-2 cells and Rat-2 mutant clones were stimulated by LPA, PDGF or PMA with or without pretreatment of 1-butanol, *t*-butanol or propranolol. Cells were washed with ice-cold phosphate-buffered saline twice and then lysed in SDS-sample buffer without 1,4-dithiothreitol. Harvested cell lysates were boiled and the protein concentration was measured using BCA method. Up to 10 μg of cell lysate was loaded on the 4–20% gradient SDS–polyacrylamide gels and analyzed by Western blotting performed with appropriate antibodies.

PLD activity measurement. Rat-2 cells were starved in DMEM for 24 h in the presence of 1 μCi/ml of [9,10-^{3}H]myristic acid. Cells were washed with DMEM three times and incubated in fresh DMEM for 1 h. After a pretreatment of 0.3% 1-butanol for 5 min, cells were stimulated with LPA, PDGF or PMA for various times indicated and the formation of [^{3}H]PtdBuOH was measured as previously described [18].

Results and discussion

PA produced by PLD is required for PKD phosphorylation

Since PA production by PLD can be inhibited by 1-butanol but not by *t*-butanol, 1-butanol is commonly used to examine the possible PLD requirement for cellular processes. In order to examine the requirement of PLD for PKD activation, we measured the effect of butanol on the auto-phosphorylation of PKD at Ser916 which is an indicator of PKD activation [4]. As shown in Fig. 1A (upper panel), pretreatment of Rat-2 fibroblasts with 0.5% 1-butanol reduced the auto-phosphorylation of PKD induced by LPA stimulation, whereas pretreatment with *t*-butanol did not. Interestingly, the PKC-dependent trans-phosphorylation at Ser744/748 was also inhibited by 1-butanol (Fig. 1A, middle panel). This latter inhibitory effect suggests that even PKC-mediated phosphorylation requires PLD activation. This result supports the sequential activation model for PKD suggested by Hausser et al. [5]. According to the model, PKD translocation to target membrane and auto-phosphorylation precede trans-phosphorylation by PKD, and DAG binding plays an essential role in the early step of translocation [2]. The results of Fig. 1A show that both auto-phosphorylation and PKC-mediated phosphorylation require PLD action and imply that PLD is required for an early step of PKD activation.

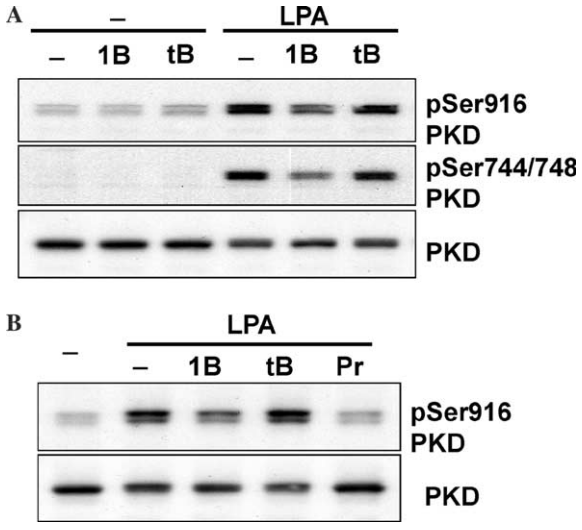

Fig. 1. PA production by PLD is required for PKD activation by LPA. (A) Rat-2 cells were starved for 24 h and pretreated with 0.5% 1-butanol (1B) or *t*-butanol (tB) for 10 min. The phosphorylation of PKD at Ser916 (upper panel) or Ser744/748 (middle panel) was monitored by Western blotting using antibodies specific to phospho-Ser916 or phospho-Ser744/748. The total amount of PKD was visualized using a PKD-specific antibody (bottom panel). (B) Rat-2 cells were pretreated with 100 μM propranolol (Pr) for 1 min and stimulated by 10 μM LPA for 5 min. The phosphorylation of PKD at Ser916 was compared with that in cells pretreated for 10 min with 1- or *t*-butanol.

LPA-induced PKD activation is mediated by DAG elevated by PLD signaling

It has been reported that DAG plays an essential role in the recruitment of PKD to the *trans*-Golgi network [2]. Since PA produced by PLD can be metabolized to DAG by PA phosphohydrolase, and propranolol can

inhibit this reaction, we examined the effect of propranolol on LPA-induced PKD phosphorylation. As shown in Fig. 1B, brief pretreatment with propranolol for 1 min blocked LPA-induced PKD phosphorylation at Ser916 to a greater extent than 1-butanol. This result supports the idea that PLD-mediated PA formation is involved in LPA-induced PKD activation by increasing DAG. Propranolol also can block translocation of PKD to the Golgi [2]. This result also supports the sequential activation model which suggests a close relationship of DAG binding to auto-phosphorylation.

PMA-induced PKD activation is PLD-independent

The effect of 1-butanol on PKD activation by platelet-derived growth factor (PDGF) and 4β-phorbol 12 myristate 13-acetate (PMA) was monitored since these agents can activate both PLD and PKD [1,10]. PDGF induced PKD auto-phosphorylation, but the magnitude of the effect was much less than that of LPA (Fig. 2 compared with Fig. 1). The phosphorylation was sensitive to 1-butanol, but the inhibition was also less than that seen with LPA. In contrast to LPA and PDGF, PMA-induced PKD auto-phosphorylation was not affected by 1-butanol. This is consistent with an effect of 1-butanol to inhibit DAG production via PLD, but not to impair the action of PMA.

Since it is hard to exclude a possible artifact of 1-butanol as an alcohol, we compared PKD phosphorylation in wild type Rat-2 cells and in two mutant clones, Rat2V25 and Rat2V29, that exhibit reduced PLD activity [18]. The increase of auto-phosphorylation induced by LPA or PDGF was clearly reduced in the two mutant clones compared with the wild type Rat-2 cells (Fig. 3). The phosphorylation at Ser744/Ser748 also showed a similar difference between wild type and mutant cells. These results agree well with the effects of 1-butanol in Fig. 1 and support the hypothesis that PLD activation is essential for PKD activation. In agreement with the results of Fig. 2, PMA-induced phosphorylation was not reduced in the mutant cells. These results again showed that the PMA signal does not require PLD action for PKD activation due to its DAG-mimicking effect. We obtained similar results to those with PMA using 1,2-dioctanoyl-*sn*-glycerol (DOG), a cell permeant form of DAG (data not shown). These results support the concept that PMA and DOG mimic the DAG effect and that PLD is required for the effects of LPA and PDGF because it can increase the DAG level.

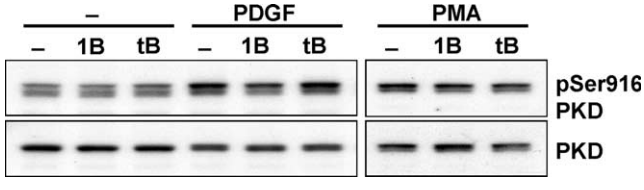

Fig. 2. PLD activity is required for PDGF-induced PKD activation but not for PMA-induced activation. Rat-2 cells were starved and stimulated with 50 ng/ml PDGF or 100 nM PMA for 10 or 15 min, respectively, with or without pretreatment with 1-butanol or *t*-butanol. The phosphorylation of PKD at Ser916 and the total PKD amount were visualized as described in Fig. 1.

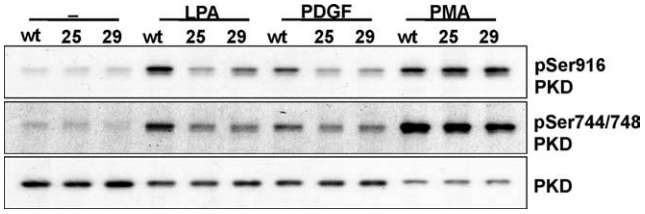

Fig. 3. PKD activation in PLD activity-reduced cell lines. Wild type Rat-2 (wt) and Rat-2 mutant clones, Rat2V25 (25) and Rat2V29 (29), were starved and stimulated with 10 μM LPA, 50 ng/ml PDGF or 100 nM PMA for 5, 10 or 15 min, respectively. Cells were lysed and phosphorylation of PKD at Ser916 and Ser744/748 was compared by Western blot.

It has been reported that DOG treatment induces translocation of PKD to the plasma membrane where DOG is more richly distributed than the Golgi [2]. However, DOG still induced PKD phosphorylation (data not shown). Therefore, PKD targeting to a proper location seems to be more important for precise regulation of PKD function. Local elevation of DAG through PLD could play a key role in this regulation. Since PLD1 has been reported to be localized in perinuclear vesicular structures including the Golgi (Exton 2002), LPA-induced PLD activation might be involved in regulating PKD function in the Golgi under physiological conditions.

Temporal differences in LPA, PDGF, and PMA-induced activation of PLD and PKD

In order to get further information about the correlation between PLD activation and PKD phosphorylation, their temporal changes after stimulation by three different agents were compared. The phosphorylation of PKD Ser916 was increased by LPA, PDGF, and PMA, as expected (Fig. 4A). PKC-dependent phosphorylation at Ser744/Ser748 was also increased by the three stimuli. However, the two types of PKD phosphorylation showed different temporal patterns. First, even though PKC-mediated phosphorylation of PKD has been suggested to be essential for PKD activation, the phosphorylation at Ser744/Ser748 was a slower event compared with the phosphorylation at Ser916, which indicates PKD activation. Maximum phosphorylation at Ser744/Ser748 was obtained only after 15 min treatment with LPA, PDGF or PMA, whereas phosphorylation of Ser916 was achieved much sooner (Fig. 4A). The three agonists showed different temporal patterns of PKD auto-phosphorylation at Ser916 and

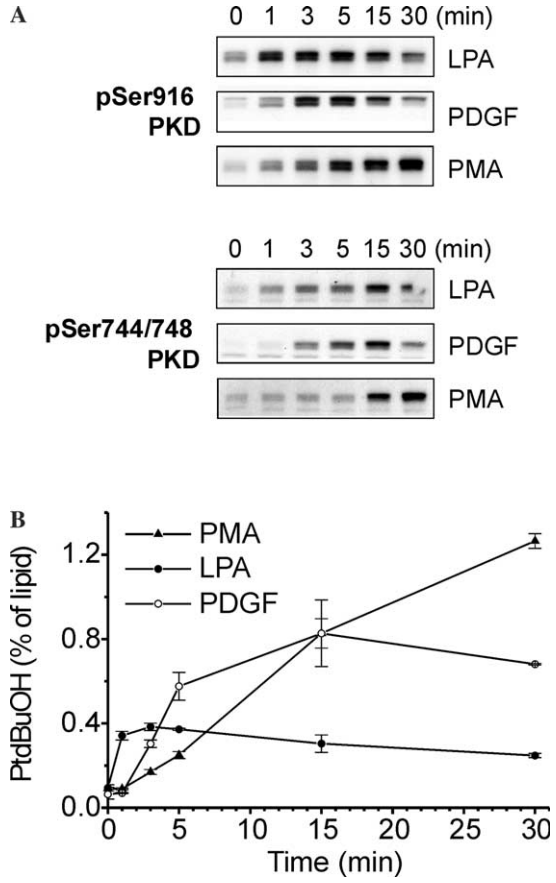

Fig. 4. Temporal patterns of PKD phosphorylation and PLD activation. (A) Rat-2 cells were stimulated with 10 μM LPA, 50 ng/ml PDGF, or 100 nM PMA for the times indicated and lysed. Phosphorylation of PKD at Ser917 and Ser744/748 visualized by Western blotting. (B) Rat-2 cells were starved for 24 h in the presence of [9,10-^{3}H]myristic acid. Cells were pretreated with 0.3% 1-butanol for 5 min and stimulated by 10 μM LPA, 50 ng/ml PDGF, or 100 nM PMA for the times indicated. PLD activity in each sample was measured as described in Methods. Mean ± SE values of the radioactivity in PtdBuOH expressed as a percentage of total lipid radioactivity are shown.

PKC-dependent phosphorylation of Ser744/Ser748 (Fig. 4A). LPA induced a rapid increase (within 1 min) in both phosphorylations while the PDGF effect was slower and declined after 15 min. The PMA effect developed more slowly and was sustained through 30 min.

Interestingly, the temporal patterns of PKD phosphorylation at Ser916 resembled the patterns of PLD activation. As shown in Fig. 4B, LPA induced the most rapid increase of phosphatidylbutanol (PtdBuOH) formation by PLD activity with a near-maximal level within 1 min. PDGF and PMA caused an increase after a 1 min delay. Thereafter, PDGF induced a more rapid accumulation of PtdBuOH than did PMA, but this ceased after 15 min. The slower increase with PMA persisted through 30 min. These results suggest a close relation between PLD and PKD activation by three different stimuli and support the requirement of PLD for PKD auto-phosphorylation. Since PKC-mediated phosphorylation of PKD at Ser744/Ser748 was a later event than auto-phosphorylation at Ser916, it also strongly supports the sequential PKD activation model and suggests PLD is required for the first step.

Discussion

Although the present results implicate PLD in the regulation of PKD by LPA, they suggest that DAG rather than PA is the actual regulator of PKD. This is based on the effects of a synthetic DAG and of an inhibitor of the conversion of PA to DAG. The question arises, therefore, as to why PLC, which directly generates DAG and is also activated by LPA [13], is not the more important regulator of PKD. A possible reason is that DAG formation by PLC is transient and limited in magnitude. Many studies [19] have shown that in agonist-stimulated cells, there is a brief peak of DAG derived from PLC action on phosphoinositides accompanied by a larger, more prolonged increase in DAG derived from phosphatidylcholine via PLD-generated PA.

In addition to this quantitative issue, there is the question of the different cellular locations of DAG derived from phosphoinositides vs phosphatidylcholine. LPA-induced generation of DAG via PLC occurs in the plasma membrane since this is where the receptors, G proteins and PLC, are located. In contrast, the regulated isozyme of PLD (PLD1) is mainly localized to the perinuclear region including the Golgi apparatus [10,20,21]. This suggests that DAG produced by PLD in response to LPA will be generated at or near the Golgi, i.e., the site to which PKD is translocated and activated in a DAG-dependent manner [2,5].

To explain the observation that auto-phosphorylation of Ser916 in PKD precedes PKC-mediated transphosphorylation of Ser744/Ser748 (Fig. 4A), one would have to postulate that the activation of PLD in the Golgi by LPA occurs more rapidly than the activation of PKC at this site. Since present methodology precludes defining accurate time courses of activation of these two enzymes in the Golgi, one can only speculate about this possibility. Fig. 4B shows that LPA activates PLD more rapidly than do PDGF and PMA. Studies of Rho activation by LPA in many cell types and the utilization of Clostridial toxins have shown that Rho is activated very rapidly by this agent and is an important mediator of PLD1 activation [10]. Furthermore, Yuan et al. [15,16] have provided strong evidence that G_{13} and Rho are involved in the activation of PKD by G protein-coupled agonists. On the other hand, PLD activation by PMA and PDGF is significantly slower than that induced by LPA (Fig. 4B). Since PKC is undoubtedly the mediator of PMA and plays the major role in the PDGF effect [7]

and since Yuan et al. [22] have demonstrated that G_q and PKC also play a role in the activation of PKD, these results suggest that the activation of PKC or its translocation to the intracellular site of PLD1 is not as rapid as for Rho. In other words, Rho-mediated activation of PLD1 by LPA could rapidly generate DAG at the Golgi causing autophosphorylation of PLD, whereas PKC activation and translocation to this site might be slower, resulting in a slower trans-phosphorylation of PKD.

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