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Propranolol increases phosphatidic acid level via activation of phospholipase D[1]

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KEY WORDS phospholipase D; phosphatidic acids; protein kinase C; propranolol; staurosporine; A-549 cells; immunoblotting

ABSTRACT

AIM: To investigate the propranolol-induced phospholipase D (PLD) activity, its contribution to the increase in the level of phosphatidic acid, and the role of protein kinase C (PKC) in this event. **METHODS**: A combination of [^{3}H]-myristate labeling, transphosphatidylation reaction, lipid extraction, and thin layer chromatography was used to measure the PLD activity. PKC inhibitors and prolonged phorbol-12-myristate-13-acetate (PMA) treatment were used to study the involvement of PKC in propranolol-induced PLD activation. Immunoblotting was used to detect the intracellular levels of PKC. **RESULTS**: Treatment of A-549 cells with propranolol in the presence of butanol, resulted in the rapid activation of PLD. Propranolol induced the formation of phosphatidylbutanol (PBut), a unique product of PLD, at the expense of phosphatidic acid (PA) formation. Pretreatment of cells with PKC inhibitors Ro-31-8220, staurosporine, and rottlerin increased the propranolol-induced PLD. Down-regulation of PKC by prolonged treatment of cells with PMA also potentiated the propranolol-induced PLD activity. **CONCLUSION**: Propranolol induces rapid activation of PLD activity, which results in the increase in intracellular level of PA. The data also indicate that propranolol-induced PLD activity could be negatively regulated by PKC.

INTRODUCTION

Regulation of the production of intracellular second messengers via different signaling pathways has been known as an indispensable signaling event elicited by many extracellular stimuli[1,2]. Phosphatidylcholine (PC)-specific phospholipase D (PLD) catalyzes the hydrolysis of PC, which generates choline and phosphatidic acid (PA). PA can be hydrolyzed by PA phosphatase to generate diglyceride (DG), a physiologic activator of PKC. PA itself has been known as an intracellular second messenger that is important in the regulation of some biological events[3]. PA can also be generated through the phosphorylation of DG by DG kinase[4,5]. In the presence of a primary alcohol, PLD catalyzes the formation of phosphatidylalcohol, which is a unique product of PLD[6]. The examination of the intracellular DG and PA levels or the product of transphosphatidylation reaction has been used to investigate the lipid signaling pathways and the involvement of PC-specific PLD.

Regulation of PLD activity by PKC has been intensively studied in the past several years. The activation of PLD activity induced by phorbol-12-myristate-13-acetate (PMA) can be easily detected in vast variety of cells and tissues. Phorbol ester is probably the strongest exogenous stimulant used for eliciting PLD activity in intact cells, suggesting that PKC is a major stimulator for PLD activity. PKC-dependent and PKC-independent activation of PLD had been reported in the past several years[7-19].

Propranolol, which has been used clinically as a beta-adrenergic receptor antagonist, has been reported to have an inhibitory effect on the PA phosphatase activity. This effect was considered to be the cause leading to the elevated PA amount in intact cells treated with propranolol[20-23]. By exploiting this observation,

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propranolol has been used in studies on the intracellular signaling events associated with the DG and PA formation. Some conclusions regarding the roles of DG and PA or their involvement in cellular processes were therefore drawn according to the effects induced by propranolol. However, the inhibition of PA phosphatase by propranolol detected *in vitro* as reported may not reflect the case in intact cells. Kiss[24] reported several years ago that PLD could be activated at a specific time point after the propranolol treatment in NIH 3T3 fibroblasts. In an irrelevant experiment, we found that propranolol elicited strong increase in PLD activity in A-549 cells.

To obtain a better understanding on the effect of propranolol and the possible mechanism related with the propranolol-induced PLD activity, we investigated in more details the relationship of propranolol-induced PLD activity with intracellular PA accumulation, and the involvement of PKC in propranolol-induced PLD activation.

MATERIALS AND METHODS

Materials A-549 (Human lung epithelial cancer cells) was obtained from ATCC (American Type Culture Collection, USA). Cell culture reagents and F-12K medium were purchased from GIBCO (USA). [9, 10-^{3}H]-Myristic acid (1.813 GBq/mol), En^{3}hance spray was purchased from New England Nuclear (NEN) Life Science Products (Boston, MA, USA). Phosphatidylbutanol (PBut) standard was from Avanti Polar Lipids (Alabaster, AL, USA). *dl*-Propranolol was from Sigma (St Louis, MO, USA). 1-Butanol was from Aldrich Chemical Company, Inc (Milwaukee, WI, USA). Silica-gel 60 Å thin layer chromatography (TLC) plates were from Whatman (Clifton, NJ, USA). Ro-31-8220, PMA, and rottlerin were from CALBIOCHEM® (San Diego, CA, USA). Rabbit polyclonal antibodies against PKC-α, -β_{II}, -δ, and -ϵ were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Enhanced chemiluminescent reagents and nitrocellulose membrane were from Amersham Life Science (Buckinghamshire, England). All other reagents were from Sigma unless otherwise indicated.

Cell culture and labeling A-549 cells were cultured in 60-mm plates at 37 ℃, under 5 % CO_2, in F-12K medium supplemented with 10 % newborn calf serum (NCS) and penicillin (100 kU/L)/streptomycin (100 mg/L). At 80 % – 90 % confluence, cells were made quiescent by incubating in 1.5 mL of F-12K medium containing 0.5 % NCS for 24 h and then labeled with [^{3}H]-myristic acid (0.111 MBq each plate) overnight before the addition of indicated agents. PLD activity was determined as measured by the formation of PA and/or phosphatidylbutanol (PBut). For measurement of PBut, cells were treated with or without indicated agents in the presence of 1-butanol (0.4 %, final concentration). The reactions were stopped and the lipids were extracted and analyzed as described below.

Lipid extraction and analysis Lipids were extracted and analyzed as previously described[25] with minor modifications. Reactions were stopped by quickly pouring off the medium and treating the cells with 600 μL of ice-cold CH_3OH/HCl 6 mol/L (50 : 2, vol : vol). Samples were scraped and moved into Eppendorf tubes and extracted with the same volume of ice-cold $CHCl_3$ and 200 μL of NaCl solution (1 mol/L). The organic phase was transferred to another Eppendorf tube and re-extracted with ice-cold NaCl (0.35 mol/L, 600 μL) and CH_3OH/HCl 6 mol/L (50:2, vol:vol, 200 μL). The organic phase was dried by vacuum concentrator. Extracted lipid samples were redissolved in 30 – 40 μL of $CHCl_3/CH_3OH$ (95 : 5) and applied to silica-gel 60 Å thin layer chromatography plate. The lipids were separated by different solvent systems: for PA and PBut, the upper phase of ethylacetate-isooctane-acetic acid-water (90 : 50 : 20 : 100, solvent system Ⅰ); for DG and monoacylglyceride (MG), hexane-diethylether-methanol-acetic acid (90 : 20 : 3 : 2, solvent system Ⅱ). PA, PBut, MG, and DG were separated twice with solvent system Ⅰ. For each separation, chromatography was stopped when the solvent front reaches the middle of the TLC plate. The samples were then further separated once with the solvent system Ⅱ. The plate was lightly stained with iodine vapour and the standard lipids were marked. Plate was sprayed with En^{3}hance spray, wrapped with Saran Wrap, and exposed by autoradiography with a Kodak film at – 80 ℃. Specific lipids were identified with the references of the migration of lipid standards and were scrapped from TLC plates into scintillation vials. Lipids were eluted out of the silica-gel by adding 0.5 mL CH_3OH and then mixed with 2.5 mL Biodegradable Counting Scintillant (BCS, Amersham), and the radioactivity was measured by liquid scintillation spectrometry.

Cell lysis and immunoblot The procedures described by Brodie *et al*[26] were followed with minor

modifications. Cells were washed with ice-cold PBS, lysed with 1 mL lysate buffer [Tris 50 mmol/L pH 7.4, NaCl 100 mmol/L, edetic acid 2 mmol/L, 1% Triton X-100, phenylmethylsulfonyl fluoride (PMSF) 1 mmol/L, aprotinin 5 mg/L, and leupeptin 5 mg/L]. The lysates were homogenized 3 times with a 25-gauge needle and incubated on ice for 30 min before centrifuged at 11 000 × *g* for 30 min. Protein concentration in each fraction was determined using Bio-Rad protein assay reagent. PKC levels were measured through immunoblotting. Briefly, lysates (80 μg of protein) were subject to SDS-PAGE (8 %) and transferred to nitrocellulose membranes (Hybond™ ECL™). The membrane were blocked with 5 % skim milk in TBS-T (Tris 10 mmol/L pH 7.5, NaCl 100 mmol/L, 0.1 % Tween-20) and subsequently incubated with primary antibody (0.1 mg/L) in TBS-T. Specific reactive bands were detected using a horseradish peroxidase conjugated goat anti-rabbit IgG (Amersham Life Science). The immunoreactive bands were visualized by enhanced chemiluminescent reagents.

PKC down-regulation and protein kinase inhibitor treatment Cells were incubated in 2.0 mL of F-12K containing 0.5 % NCS. For determination of PKC level before and after prolonged PMA treatment, 90 % cells were incubated for 36 h in the absence or presence of PMA (600 μg/L), and then lysed as described[20]. For the measurement of PLD activity before and after the PKC down-regulation, cells were treated with PMA (600 μg/L) for 24 h, then labeled with [^{3}H] myristic acid (0.111 MBq each plate) overnight under the same condition. The medium was then removed and replaced with 1 mL fresh medium containing 0.5 % NCS. Cells were further incubated for 1 h before adding the indicated agents. To examine the effects of PKC inhibitors on PLD activity, cells were pretreated with protein kinase inhibitors 15 min prior the addition of propranolol. For measuring the PLD activity by transphosphatidylation reaction, 1-butanol (0.4 %, final) were added 5 min before adding the propranolol.

Statistic analysis Results are presented as $x \pm s$. For statistical analysis, t test was used. Differences were considered significant at a level of $P < 0.05$.

RESULTS

Propranolol induces the PLD activity To identify the mechanism through which propranolol increases the intracellular PA level, we examined the effect of propranolol on PLD activity and its relationship with increased PA formation. Fig 1 shows that PLD

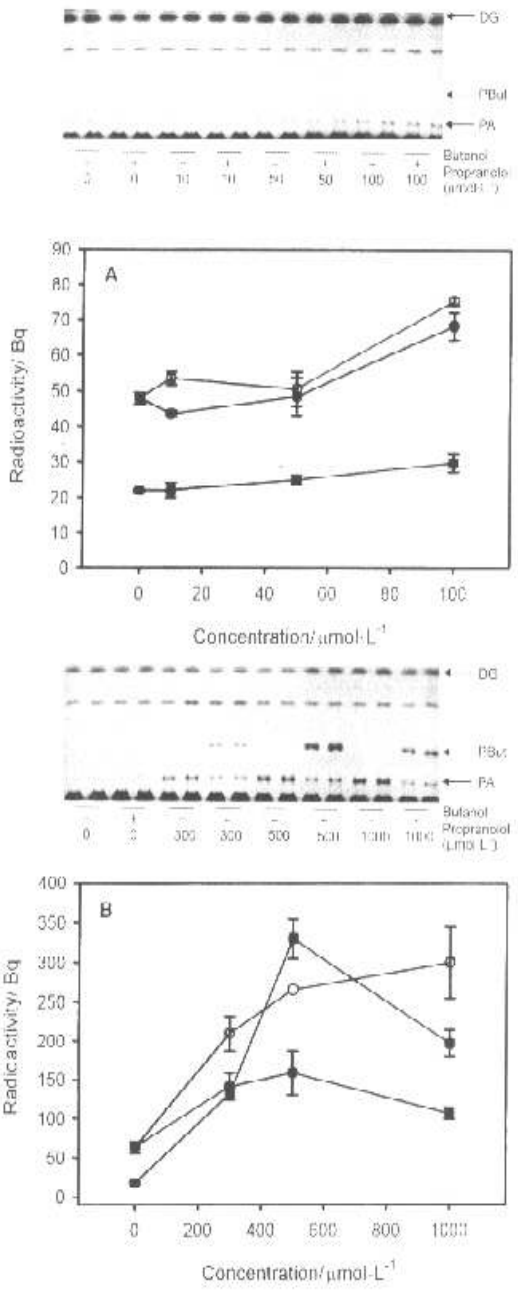

Fig 1. PLD activity induced by propranolol. A: Activation of PLD by propranolol (10 – 100 μmol/L) for 40 min. Tops: autoradiogram of the results. B: Activation of PLD by propranolol (300 – 1000 μmol/L) for 40 min. Top: autoradiogram of the results. The data shown are from one representative of three independent experiments done in duplicates. ○, PA measured in the absence of butanol; ●, PA measured in the presence of butanol; ■, PBut measured in the presence of butanol. $n = 3$. $x \pm s$.

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activity is elevated in response to relative higher concentration of propranolol in A-549 cells as measured by the increased PBut and PA formation. When cells were treated with propranolol for 40 min, a concentration-dependent increase in PBut and PA were observed as shown in Fig 1. Propranolol-induced PBut formation and increased PA production become detectable at the concentration of 100 μmol/L (Fig 1A), whereas, maximum stimulation of PLD activity occurred at 500 μmol/L (Fig 1B). However, cells started to detach from the plates at this concentration in the presence of butanol, probably due to the toxicity of PBut accumulated. As shown in Fig 1, propranolol-induced increases in PA levels were markedly reduced in the presence of butanol. The results indicate that propranolol induces the PBut generation at the expense of PA formation, suggesting that propranolol-induced PLD activation accounts for the increased PA level. Fig 2 shows that propranolol, at the concentration of 300 μmol/L, induces a rapid activation of PLD, which becomes pronounced at 10 min. Since the metabolic conversion of the PBut in cells is slow, treatment of cells with propranolol in the presence of butanol leads to a gradual accumulation of the PBut. There is no reduction during the whole period of incubation (4 h). The increase of PA formation peaked at 2 h after the propranolol treatment. In the presence of butanol, propranolol-stimulated PA formation at different time points was also reduced as compared with that in the absence of butanol.

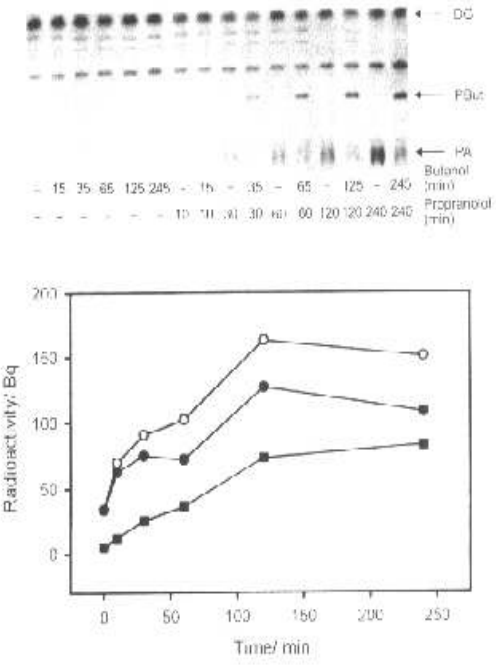

Fig 2. Time course of propranolol-induced PLD activity. Cells were treated with propranolol (300 μmol/L) for the time indicated. The PLD activity was determined as measured by the formation of PBut and PA. The data shown are from one representative result of at least four experiments. Tops: autoradiogram of the results. ○, PA measured in the absence of butanol; ●, PA measured in the presence of butanol; ■, PBut measured in the presence of butanol.

PKC inhibitors increase the propranolol-induced PLD activation We first examined the effect of staurosporine or Ro-31-8220 on the PMA-stimulated PLD activity (Fig 3A). These inhibitory effects of PKC inhibitors on PMA-induced PLD are used as an indication for their effectiveness in the inhibition of PKC. In Fig 3B, we examined the effects of PKC inhibitors on the propranolol-stimulated PLD activity. Cells were pretreated with PKC inhibitors for 15 min and then stimulated with propranolol for additional 40 min. As shown in Fig 3B, propranolol-induced PBut formation was enhanced by staurosporine or Ro-31-8220. In addition to the staurosporine and Ro-31-8220, we also investigated the effect of rottlerin, which is a specific inhibitor to isoform δ, on the PMA and propranolol induced PLD activity. rottlerin can inhibit the PMA-induced PLD activity while it potentiate the propranolol induced PLD activity by about 2 folds (Fig 3C). This result indicates that PKC-δ is involved in the regulation of propranolol-activated PLD activity. Treatment of cells with PKC inhibitors staurosporine, Ro-31-8220, and rottlerin under these conditions used to study the propranolol-induced PLD activity does not cause the obvious change of the basal level of PLD activity, which also excluded the possibility of addictive effect by these compounds at this time point. These results indicate that propranolol activates a PLD activity that is inhibited by PKC.

PKC down-regulation increases propranolol-stimulated PLD activity PKC down-regulation could be achieved through prolonged treatment of cells with PMA. We measured the total levels of PKC-α, -β_{II}, δ, and -ε in A-549 cells before and after PKC down-regulation by prolonged treatment with PMA. As shown in Fig 4A, prolonged treatment of cells with PMA depleted most of the α, δ, and ε PKC isozymes. No obvious change in total PKC-β_{II} level was observed. These results were also confirmed by measuring the PKC

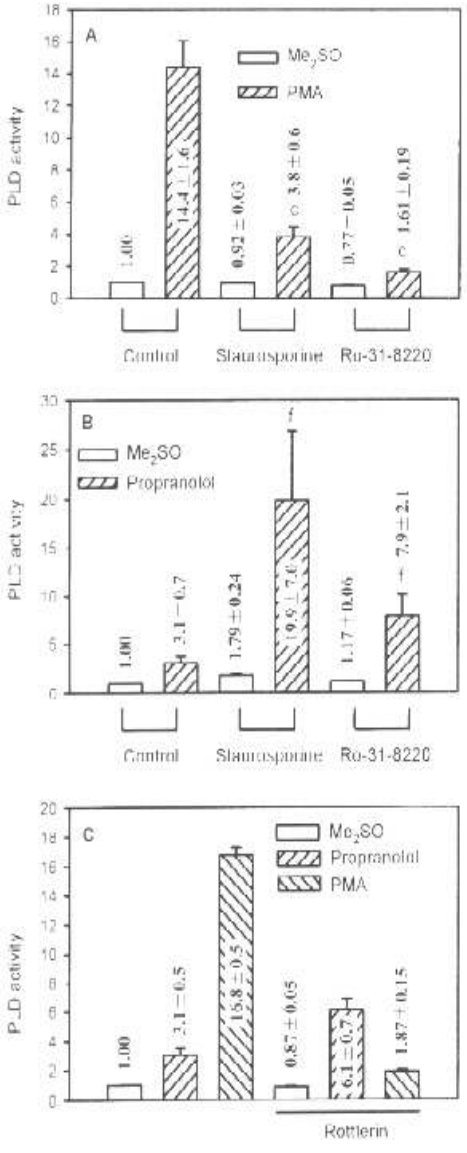

Fig 3. PKC inhibitors increase the propranolol-induced PLD activity. Quiescent A-549 cells were preincubated with staurosporine (0.5 μmol/L), Ro-31-8220 (5 μmol/L), or rottlerin (15 μmol/L) for 15 min and then stimulated with propranolol (300 μmol/L) or PMA (100 μg/L) for additional 40 min. The PLD activity was determined as measured by the formation of PBut. A: Staurosporine (0.5 μmol/L) and Ro-31-8220 (5 μmol/L) decrease the PMA-induced PBut formation. B: Staurosporine (0.5 μmol/L) and Ro-31-8220 (5 μmol/L) increase the propranolol-induced PBut formation. C: The effect of rottlerin (15 μmol/L) on the propranolol- or PMA-induced PBut formation. A and B: $n = 3$ independent experiments. C: $n = 2$ independent experiments in duplicates. $x \pm s$. $^{\#}P < 0.01$ *vs* PMA-induced PBut formation. $^{\dagger}P < 0.01$ *vs* propranolol-induced PBut formation. PLD activity was expressed as fold increase compared with non-treated control.

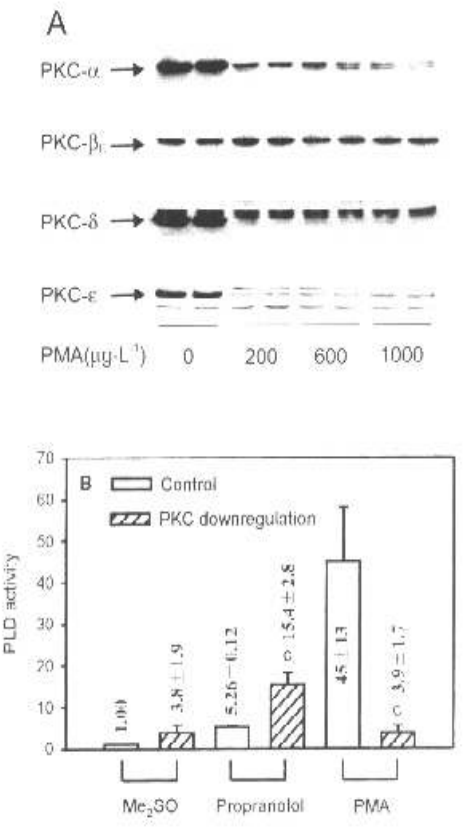

Fig 4. PKC down-regulation increases the propranolol-induced PLD activity. A: The levels of PKC isozymes before and after prolonged PMA treatment. The data shown are from one of three independent experiments with the identical results. B: PKC down-regulation increases the propranolol (300 μmol/L)-stimulated PLD activity. Prolonged PMA-treated and -untreated cells were stimulated with propranolol or PMA in the presence of 0.4 % butanol. The concentration of PMA for PLD induction was 100 μg/L. The PLD activity was determined as measured by the formation of PBut. $n = 3$ independent experiments. $x \pm s$. $^{\#}P < 0.01$ *vs* propranolol-induced PBut formation without PKC down-regulation. $^{\dagger}P < 0.01$ *vs* PMA-induced PBut formation without PKC down-regulation. PLD activity was expressed as fold increase compared with non-treated control.

levels in fractionated parts before and after prolonged PMA treatment (data not shown). The results indicate that prolonged PMA treatment is highly effective in reducing the levels of most PKC isozymes detected in A-549 cells. To further determine the effects of PKC on the propranolol-induced PLD activity, we examined both the basal and the propranolol-stimulated PLD activity before and after prolonged PMA treatment. As shown in

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Fig 4B, PKC down-regulation by prolonged PMA treatment increased the basal PLD activity and completely abolished the PMA-stimulated PBut formation. PKC down-regulation also enhanced the propranolol-induced activation of PLD activity, which further increased the PBut level from 5 to 15 folds. These data provide additional evidence supporting an inhibitory effect of PKC isozyme(s) in propranolol-induced activation of PLD.

DISCUSSION

In propranolol treated cells, PA and PBut levels continuously increase with no reduction after at least 4 h of incubation, indicating that the PLD activity is continuously activated by propranolol. The strong and persistent activation of PLD induced by propranolol clearly suggests that increased PA level in the presence of propranolol was at least a major consequence of the PLD activation stimulated by propranolol. If propranolol induces the increase in PA level in intact cells through its inhibitory effect on PA phosphatase activity as detected *in vitro*, then the PA level should not decrease substantially by the addition of butanol, and this is not the case as observed in our study. In this study, we found that the increase of PA level induced by propranolol in the absence of butanol is larger than that in the presence of butanol. The difference is substantial and can be measured at the various time points ranged from 10 min to 4 h. This evidence indicates that propranolol-induced increase in the formation of PBut was at the expense of the increased PA level, suggesting that propranolol-induced PLD activity account for the propranolol-induced increase in PA level.

The regulation of PLD activity and the production of lipid signaling molecules are important for cross-talks among different signaling pathways. The intracellular levels of lipid signaling molecules, such as DG, PA, lysophosphatidic acid (LPA), and their metabolic derivatives could be changed via alterations in the PLD activity. Both PKC-dependent and -independent activation of PLD in response to growth factors or other extracellular agonists has been reported by a number of laboratories[7-19]. However, the mechanisms still remain to be further characterized. In this study, we found that either PKC inhibitor pretreatment or PKC down-regulation markedly increased the propranolol-induced PLD activity, indicating that PKC had an inhibitory effect on propranolol-induced PLD activity. Our data presented here suggest a new mechanism by which cells activate PLD activity. It was previously reported[27] that v-Src activated a PLD activity that can be distinguished from the PLD activity activated by phorbol ester. Min *et al*[13] identified a rat brain PLD (rPLD1) and found that the phosphorylation of rPLD1 by PKC was correlated with the inhibition of its catalytic activity. Sozzani *et al*[28] reported that propranolol competitively inhibited the activity of PKC purified from human neutrophiles, and directly interacted with the regulatory domain of PKC-α and -β. Kiss *et al* reported that both sphingosine and propranolol, which had an inhibitory effect on PKC, could stimulate PLD activity[16,24]. We also found in our studies that treatment of several types of cells with sphingosine resulted in a strong activation of PLD (data not shown). These evidences imply that PKC can act as a negative regulator of PLD activity, and is of particular relevance with the finding presented. The mechanisms through which these molecules stimulate PLD activity are not well understood. One possibility is that these molecules activate a pathway, which is likely inhibited by PKC. Another possibility is that these molecules inhibit PKC isozymes that act as a negative regulator of PLD activity. It is likely that the propranolol-activated PLD activity is different from the PLD activity stimulated by phorbol esters. The activation of PLD by propranolol and PMA may involve different PKC isozymes, which have the opposite effects on PLD activity. The facts that PMA induces the rapid membrane translocation of PKC-α in A-549 cells (data not shown) suggest that these PKC isozymes were implicated in the PMA-induced activation of PLD but were unlikely implicated in the regulation of propranolol-induced PLD activity. PKC-β_{II} is also unlikely involved in the negative regulation of propranolol-induced PLD activation, because prolonged treatment of cells with PMA does not lead to the decrease of basal level of PKC-β_{II}. As well as our result showed the involvement of PKC-δ, Kiss *et al*[29] reported recently that overexpression of PKC-ε and its regulatory domain in fibroblasts inhibited the PMA-induced PLD activity. These studies provide evidence demonstrating that some PKC isozymes act as negative regulators of PMA-induced PLD activity. Determination of the specific PKC isozyme(s) and the mechanisms through which PKC inhibits PLD activity may be necessary for a better understanding of the mechanism controlling the PLD activities and its implication in cell growth, differentiation, and other functions.

In conclusion, we demonstrate here that propranolol induces a persistent activation of PLD activity in A-549 cells, which can be enhanced by PKC down-regulation or PKC inhibitors. Propranolol-induced activation of PLD accounts for the propranolol-induced increase in the level of intracellular PA. PKC down-regulation or inhibition of PKC resulted in the increase in propranolol-induced PLD activation. The present study also explains the discrepancy between the reported evidence showing PKC-dependent and -independent activation of PLD.

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普萘洛尔通过磷脂酶 D 的活化诱导磷脂酸水平的增高[1]

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关键词 磷脂酶 D；磷脂酸类；蛋白激酶 C；普萘洛尔；星形孢菌素；A-549 细胞；免疫印迹法

目的：考察普萘洛尔诱导细胞内磷脂酶 D 的活化，分析该酶的活化与普萘洛尔刺激引起胞内磷脂酸水平升高的关系，研究蛋白激酶 C 在此过程中的作用. **方法**：[^{3}H]-豆蔻脂酸放射性标记细胞，通过转磷脂酰反应，抽提细胞脂类分子用薄层层析法分析磷脂酶 D 的产物. 用蛋白激酶 C 的抑制剂来抑制蛋白激酶 C 的活性或通过佛波酯长时间处理细胞来下调蛋白激酶 C 的水平. 以免疫印记法分析蛋白激酶 C 的含量. **结果**：在正丁醇的存在下，普萘洛尔处理 A-549 细胞引起磷脂酶 D 的活性快速增高，普萘洛尔诱导磷脂酰丁醇的生成导致磷脂酸生成水平的下降. 用蛋白激酶 C 的抑制剂星形孢菌素（staurosporine），Ro-31-8220 及粗糠柴毒素（rottlerin）预处理细胞或通过佛波酯长时间处理下调胞内蛋白激酶 C 的水平对普萘洛尔诱导的磷脂酶 D 的活性没有抑制作用，而有增强效应. **结论**：普萘洛尔引起细胞内磷脂酸水平的提高是由于普萘洛尔对磷脂酶 D 活性的强烈诱导作用而导致的，蛋白激酶 C 对该过程可起到负调节作用.

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