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Distribution of Phospholipase C Isozymes in Normal Human Lung Tissue and their Immunohistochemical Localization

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Phospholipase C(PLC) plays a central role in signal transduction and it is important in cellular growth, differentiation and transformation. There are currently ten known mammalian isozymes of PLC identified and cloned. However, there are no report of PLC distribution in human lung tissue or their significances in pulmonary diseases. Presence of various PLC isozymes in normal human lung tissue was studied from surgical specimens. PLC isozymes in tissue extracts of the lung were partially purified by successive chromatographic steps on heparin-sepharose CL-6B conventional and TSKgel heparin-5PW HPLC columns and their activities were assayed. PLC activity peaks identified in the chromatography were immunoblotted with specific antibodies against ten known mammalian PLC isozymes(PLC-β_{1-4}, -γ_{1-2}, and -δ_{1-4}). In addition, immunohistochemical staining of the lung tissue was performed to determine subcellular and histological localization of PLC isozymes. The results indicate that normal human lungs contain β_1, β_3, γ_1, and δ_1 isozymes of PLC. The order of amount present in the lung tissue was PLC-δ_1>γ_1>β_1>>β_3, in descending order. On immunohistochemistry, PLC-γ_1 was most widely distributed and was present in bronchiolar epithelium, in type I and type II pneumocytes as well as in fibroblasts of the interstitial tissue. PLC-δ_1 was present in the cytoplasm of the bronchiolar epithelium whereas PLC-β_1 was localized to the apical membranous portion of the same epithelium. PLC-β_3 was seen in the nucleus of the respiratory and alveolar lining epithelium as well as in the nucleus of lung fibroblasts.

Key Words : *Phospholipase C Isozymes, Human Lung, Immunohistochemistry*

INTRODUCTION

Many extracellular signals such as variety of hormones, growth factors and neurotransmitters induce a rapid hydrolysis of phosphoinositides via a receptor mediated process in transmembrane signal transduction. Hydrolysis of phosphatidylinositol 4,5-biphosphate(PIP_2) produces two important second messengers, namely inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 binds to its specific receptors and induces a release of calcium from intracellular storage sites such as endoplasmic reticulum(Berridge, 1987). Diacylglycerol is a

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potent activator of protein kinase C which plays a critical role in cellular growth, differentiation and transformation by phosphorylating many protein components involved in the signal transduction and transcriptional control(Nishizuka, 1986).

PLC is a crucial player in signal transduction for it catalyzes the hydrolysis of PIP_2 and subsequently generates two important second messengers(Rana and Hokin, 1990). There are at least ten distinct isozymes of mammalian PLC identified, biochemically characterized from various tissues, and their respective cDNA isolated. Comparison of the deduced amino acid sequences has indicated that PLCs can be divided into three distinct types, PLC-β, PLC-γ, and PLC-δ. Each type contains more than one isozyme and these are designated by an arabic subscript following the Greek letters such as PLC-β_{1-4}, PLC-γ_{1-2}, and PLC-δ_{1-4}(Rhee et al.,1989 ; Lee et al., 1993 ; Jhon et al., 1994 ; Lee and Rhee, 1996). The sequence homology between species is more than 90 percent. All PLC isozymes contain two distinct regions of amino acid similarities designated as X and Y domains which are believed to be either catalytic or substrate binding sites(Rhee et al., 1989). All three subtypes of PLC contain pleckstrin homology domains which is either thought to be the phospholipid or membrane binding sites(Haslam et al., 1993 ; Gibson et al., 1994 ; Parker et al., 1994 ; Ferguson et al., 1995). PLC-γ_1 and -γ_2 are distinct from the rest in having unique regions called *src* homology 2(SH2) and *src* homology 3(SH3) domains(Stahl et al., 1988 ; Suh et al., 1988b ; Emori et al., 1989). These functional modules are important in mediating protein to protein interactions and they bind specifically to phosphorylated tyrosine residues(Pawson and Gish, 1992) and proline-rich segments of the signaling proteins(Williamson, 1994 ; Pawson, 1995). PLC-β subfamily is known to be activated by G-protein coupled receptors. Alpha subunits as well as βγ subunits of the G-protein q family can activate PLC-β enzymes(Wu et al., 1992 ; Gierschik and Camps, 1994). PLC-γ isozymes are activated via receptors which have tyrosine kinase activity as seen in many growth factor receptors(Rhee, 1991) and it translocates to the cytoskeletal compartment of the cells(Yang et al., 1994). In addition, non-receptor tyrosine kinases, many of which are reported cellular proto-oncogenes, could activate and phosphorylate PLC-γ isozymes(Liao et al., 1993). Moreover, PLC-γ isozymes can also be activated via a tyrosine kinase independent pathway by a microtubule-associated protein tau, by concerted action with arachidonic acid in bovine brain cytosol(Hwang et al., unpublished).

Information regarding the pathologic significance of the various PLC isozymes is beginning to accummulate. Since the report of elevated content of PLC-γ_1 in human breast carcinomas(Arteaga et al., 1991), increased expression of PLC-γ_1 was noted in colorectal carcinomas (Noh et al., 1994) and stomach cancers(Hwang et al., 1996 ; Kim et al., unpublished). In benign diseases, such as familial adenomatous polyposis(Homma et al., 1994) and human skins in hyperproliferative conditions (Nanny et al., 1992), overexpression of PLC was also reported.

PLC seemed to be expressed differentially in various tissues reflecting the specialized functions of each tissue and provides for the diversity of signals(Lee and Rhee, 1995). However, the information regarding PLC in normal human lung tissue and their roles in pulmonary diseases is lacking. Therefore as a step prior to elucidating pathologic implications of the PLC in lung diseases and to provide basic information concerning PLC isozymes in lung tissue, we partially purified and studied PLC distribution in normal human lungs and localized them histologically by an immunohistochemical method.

MATERIALS AND METHODS

Materials

[^{3}H] PI and [^{3}H] PIP_2 were purchased from Du Pont-New England Nuclear. PI from Sigma, and PIP_2 from Boehringer Mannheim were used. The phospholipid used in the PLC activity assays such as phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine were purchased from Avanti Polar-Lipids (Alabaster, AL).

Preparation of the Total Lung Tissue Extract

Fresh frozen normal lung tissues from 5 different surgical specimens were mixed in equal portion(10g each). The specimens selected were taken from relatively young, non-smoking patients who underwent either lobectomy or pneumonectomy. Two were males and three were females. Their mean age was 29.2 years ranging from 23 to 37. The conditions included were two arteriovenous malformation, aspergilloma, bronchial carcinoid and stage I adenocarcinoma. The normality of the lung tissue was confirmed both grossly and histologically by experienced pathologists.

The specimens were thawed in 50 ml of homogenization buffer containing 10 mM Tris(pH 7.4), 1 mM

EDTA, 1 mM phenylmethylsulfonyl fluoride(PMSF), 1 mM dithiothreitol(DTT), 2 μg/ml leupeptin, 2 μg/ml aprotinin, and calpain inhibitor I and II (each at 4 μg/ml). It was then homogenized thoroughly in a glass homogenizer with a motor-driven teflon pestle and centrifuged at 1,000×g for 10 minute to remove the debris. The supernatant(45ml) was adjusted to 2 M KCl by adding solid KCl. It was then stirred for 2 hours at 4℃ to extract PLC from both the membrane and cytosol. The crude PLC extract from previous step was then centrifuged at 23,000×g for 1 hour. The resulting supernatant was dialyzed overnight against 5 liters of homogenization buffer and then centrifuged at 16,000×g for 30 minutes. The supernatant was taken and used for the next step.

PLC Activity Assay

PLC activity assays performed during the partial purification of PLC isozymes from the lung tissue were done either using [^{3}H] PI or [^{3}H] PIP_2 as substrate in either 1 μM or 1 μM free calcium concentrations as described elsewhere(Fabiato, 1988).

Briefly, PI hydrolyzing activity was measured in a 200 μl assay mixture containing 150μM PI, [^{3}H] PI (20,000 cpm), 50mM Hepes(pH 7.0), 3mM $CaCl_2$, 2mM EGTA, 0.1% sodium deoxycholate and an enzyme source. For PI hydrolyzing activity, incubation was performed at 37℃ for 5 min. PIP_2 hydrolyzing activity was measured in 100 μl of reaction mixture containing [^{3}H] PIP_2 (30,000 cpm), 30 μM PIP_2, 50 mM Hepes(pH 7.0), 2mM EGTA, 0.1% sodium deoxycholate, and 1 μM free calcium. An enzyme source was then added to the reaction mixture and incubated at 30℃ for 10 minutes.

For both methods, the lipids in chloroform were dried under a stream of nitrogen gas, resuspended in 50mM Hepes and 0.1% sodium deoxycholate by sonication. After appropriate incubation with an enzyme source as described above, the reaction was stopped by adding 1ml of chloroform : methanol : HCl(100:100:0.6, v/v/v) followed by an addition of 0.3ml of 1 M HCl containing 5mM EGTA. After a brief centrifugation, 0.5ml of the upper aqueous phase was taken and counted for ^{3}H radioactivity with liquid scintillation spectroscopy.

Heparin-Sepharose CL-6B Conventional Chromatography

The normal lung extract from the previous step was loaded onto a heparin-sepharose CL-6B column(40ml bed volume) which had been equilibrated with 50mM Hepes(pH 7.0), 1mM EGTA, 0.1mM DTT. After washing the column with three times the bed-volume of the same buffer, the protein was eluted with a step gradient of 1 M NaCl in the equilibration buffer at a flow rate of 3 ml/min. 6 ml fractions were collected and assayed for PLC activity. Fractions(15 to 23) showing PLC activity were pooled(Fig. 1), and concentrated to an approximate volume of 10ml in a stirred ultrafiltration cells fitted with a YM 30 membrane(Amicon, Danvers, MA). The

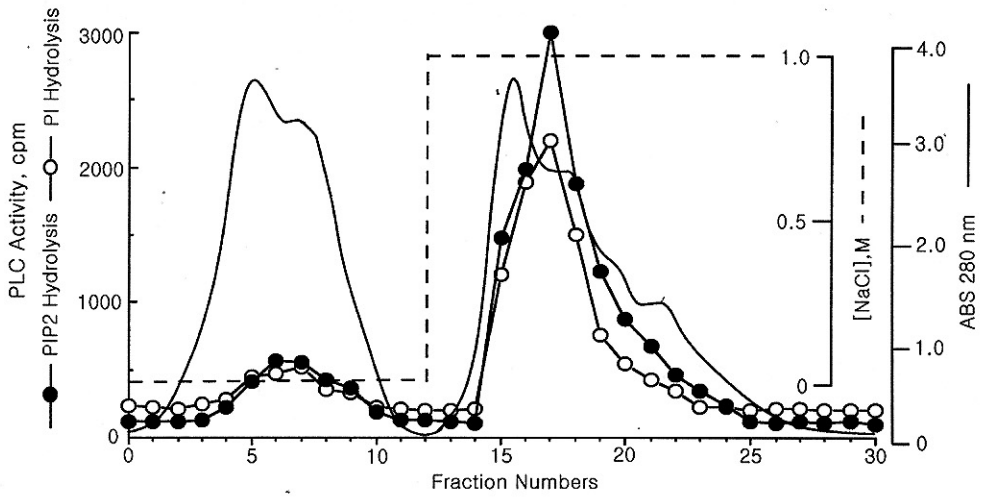

Fig. 1. Partial purification of PLC isozymes by heparin conventional chromatography. 2 M KCl extract of the lung tissue containing 200mg of protein was subjected to a chromatography on heparin-sepharose CL-6B column with a step gradient of 1 M NaCl. Fractions(6ml each) were collected and assayed for both PI and PIP_2 hydrolyzing activity.

sample was then dialyzed extensively in the same buffer and subjected to the next step.

Separation of PLC isozymes on TSKgel Heparin-5PW HPLC

A partially purified sample(20mg in protein) from the previous chromatographic step was then injected into a TSKgel heparin-5PW HPLC column(7.5×75mm, TosoHaas) that had been equilibrated with 20mM Hepes(pH 7.0), 1mM EGTA, 0.1mM DTT. Proteins were eluted, at a flow rate of 1.0ml/min, by successively applying the equilibration buffer for 15 min, a linear NaCl gradient of 0 to 0.64 M in 40 min, second linear NaCl gradient from 0.64 to 1.0 M in the next 20 min, and 1.0 M NaCl in equilibration buffer for the last 10 min of the run(Fig. 2A). Fractions(1.0ml) were collected and each fraction assayed for both PI and PIP_2 hydrolyzing activity under the described conditions. The heparin HPLC was repeated 3 times to obtain the representative results and get enough samples for the subsequent immunoblotting.

Immunoblot Analysis

Portions of each fractions from heparin HPLC showing PLC activity were treated with 4x Laemmli's sample buffer and resolved on SDS-PAGE(6% gel). Proteins were transferred to a nitrocellulose paper, incubated with various PLC isoform specific antibodies, and visualized with alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit IgG antibodies(Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD). Anti-PLC isoform antibodies used in immunoblotting were K92-3(anti-PLC-β1 monoclonal antibody), F7-2(anti-PLC-γ1 monoclonal antibody), anti-PLC-γ2 monoclonal antibody, S11-2(anti-PLC-δ1 monoclonal antibody) and others were polyclonal(Suh et al., 1988a). All of the antibodies used in the experiment were affinity purified and 1:1,000 dilution was used for the monoclonal antibodies and 1:500 dilution was used for the polyclonal antibodies. The antibodies were kindly provided by Dr.

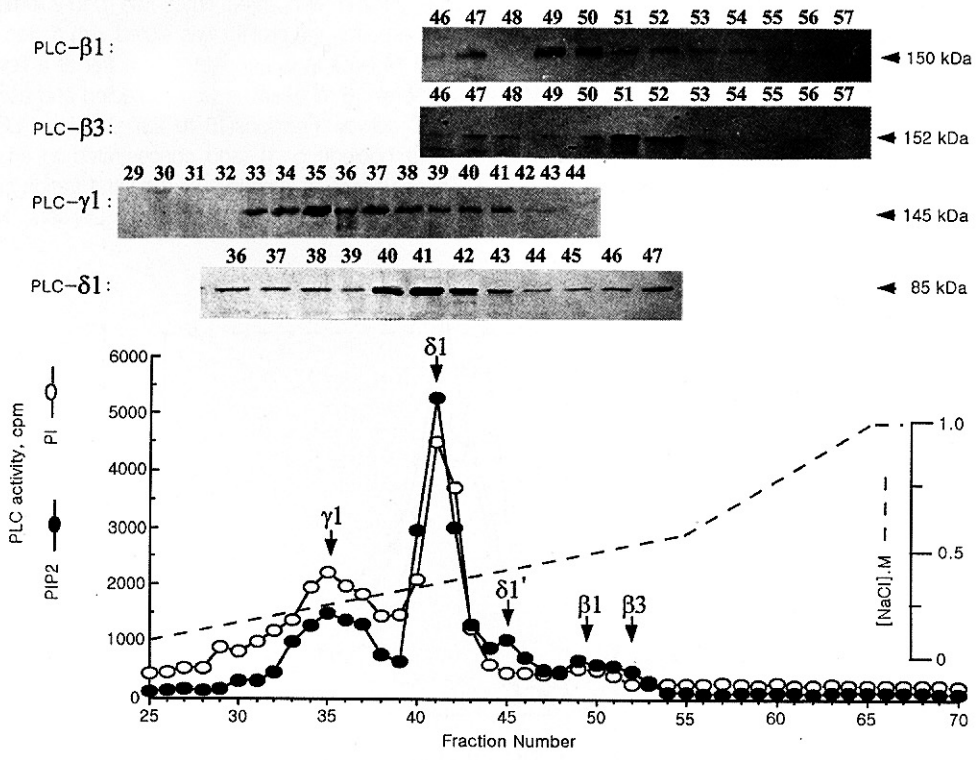

Fig. 2. Anaysis of PLC isozymes in normal human lung extracts. Pooled PLC fraction from the heparin-sepharose chromatography was resolved on TSKgel heparin-5PW HPLC. Fractions(1.0ml) were collected and assayed for both PI and PIP_2 hydrolyzing activity(*bottom*). Each activity peak identified was subjected to immunoblot analysis by specific anti-PLC antibodies as indicated on the left, and the arrows mark the molecular weight(*top*).

Sue Goo Rhee from the Laboratory of Cell Signaling, National Heart, Lung, and Blood Institute at National Institute of Health (Bethesda, Maryland, USA).

Immunohistochemistry of the Lung Tissue

Immunohistochemical staining of the paraffin sections of the normal human lung tissue was performed with each PLC isoform specific antibodies(anti-PLC-β_1 γ_1,-γ_2, and -δ_1 monoclonal antibodies and others, polyclonal). Serial 5mm sections from formalin-fixed and paraffin-embedded lung tissue were made. After deparaffinization and hydration, sections were incubated with 10% normal goat serum to block non-specific bindings. Sections were then incubated overnight at 4℃ with specific anti-PLC antibodies in either 1:800 (monoclonal antibodies) or 1:200 (polyclonal antibodies) dilution to minimize nonspecific background noises. After an overnight incubation, slides were washed three times with phosphate buffered saline and incubated with avidin-biotin reagents from LSAB kit (Dako, Denmark). As for the negative control, phosphate buffered saline or antibodies against PLC isozymes not found in the lung tissue, such as anti-PLC-γ_2 or anti-PLC-δ_4 antibodies, were used. The immunohistochemical staining was repeated three times to ensure the intensity of staining and to obtain the representative results. The sections were reacted with AEC as a chromogen and counter stained with hematoxylin. The photographs were taken under an Olympus BH2 light microscope(Olympus Optical Co., Tokyo, Japan).

RESULTS

Heparin-Sepharose CL-6B Column Chromatography

The total lung extract containing about 200mg protein was subjected to a heparin conventional column chromatography(Fig. 1). The fractions, 15 to 23, showing PLC activity were pooled and concentrated for the next step(about 70mg of protein). As determined from activity counts in the crude extract and pooled fractions, it was estimated that more than 90% of PLC activity that was present in the crude extract was recovered in the pooled PLC fractions and the loss was considered to be minor.

PLC Isozymes in Human Lung Tissue

Pooled PLC fractions from conventional heparin column chromatography were fractionated on heparin-5PW HPLC chromatography. Each fraction was assayed for PLC activity. Four main PLC activity peaks were identified. Two prominent peaks of PI-hydrolyzing acitvity were centered around fraction 35 and 41, and a small but definite peak was also detected around fraction 49(Fig. 2, bottom). For PIP_2 hydrolysis as the assay, two major activity peaks centered at fraction 35 and 41 were noted. The minor broad peaks at fraction 45, 49 and 52 were also observed(Fig. 2, bottom). The fractions showing the PLC activity were immunoblotted with isozyme specific antibodies to PLC-$\beta_{1\text{-}4}$, -$\gamma_{1\&2}$, and -$\delta_{1\text{-}4}$(Fig. 2, top).

Immunoblotting results indicated that the activity peak centered around fraction 35 was attributable to PLC-γ_1(145 kDa). Another major peak on fraction 41 was found to be PLC-δ_1(85 kDa) with a part of it eluting at fraction 45 as a minor peak(Fig. 2). PLC-β_1(150 kDa) peak was rather broad and was centered around fraction 49. A small yet definite PLC-β_3(152 kDa) peak was also observed and the intensity was maximal at fraction 51(Fig. 2). PLCs recognized by other isozyme specific antibodies such as PLC-β_2, -β_4, -γ_2, -$\delta_{2\text{-}4}$ were not present in the lung preparations, suggesting that these enzymes were either absent or present in very low amount in normal human lung to be detected by such methods.

The estimated amounts of PLC enzymes present in the lungs measured by comparing the immunoblotting intensity to a known standard were PLC-δ_1(about 400 ng/g of lung tissue), > PLC-γ_1(200ng/g) >> PLC-β_1(50 ng/g) > PLC-β_3(20ng/g) in descending order.

Immunohistochemical Localization of PLC-β_1, -β_3, -γ_1, and -δ_1 in the Lung Tissue

Immunohistochemistry of the PLC isozymes found in the lungs was performed on formalin-fixed paraffin sections of the normal lungs. PLC-γ_1 seemed to be universally present in the lung tissues and it was observed in the bronchiolar and alveolar lining epithelium as well as fibroblasts found in the interstitial tissue (Fig. 3A and 3B). PLC-γ_2, although structurally similar to PLC-γ1, was not found in any of the cells of the same slides and it was shown as a negative control(Fig. 3C). PLC-δ_1 was localized mainly to the cytoplasmic portion of epithelial cells of the bronchiole and alveolar lining cells(Fig. 3D). PLC-β_1 was localized to the apical membranous portion of the bronchiolar epithelium, which correlates well with the data which indicates that a major fraction of PLC-β is found in the membrane compartment(Fig. 3E). PLC-β_3, although found in relatively

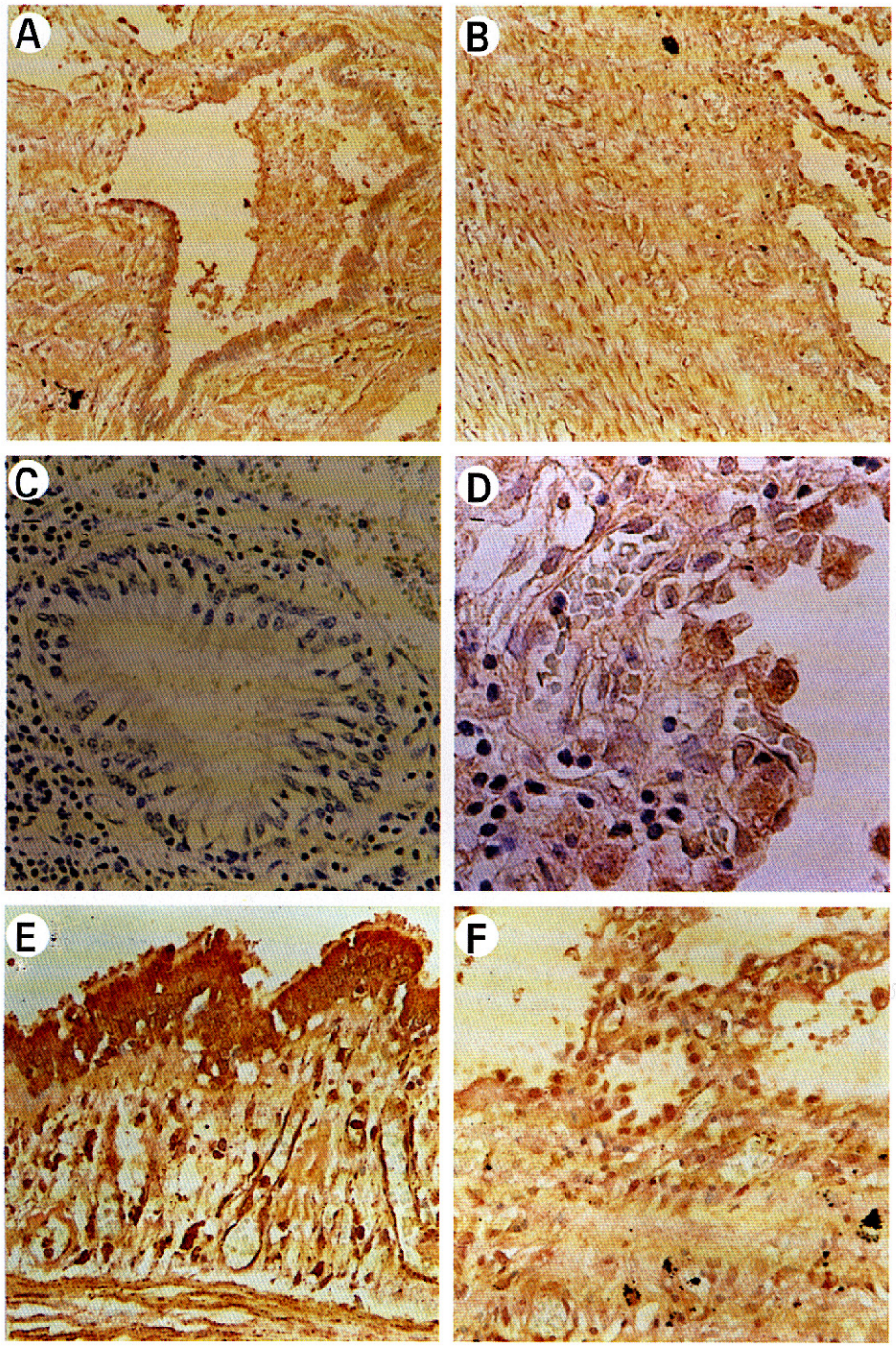

Fig. 3. Immunohistochemical Localization of Various PLC Isozymes in the Lung Tissue. PLC-γ_1 stain is seen in the cytoplasms of bronchiolar and alveolar epithelial cells and lung fibroblasts(A and B, ×100) whereas PLC-γ_2 is shown as a negative control(C, X200). PLC-δ_1 is also seen in the cytoplasms of bronchiolar epithelium(D, ×400). PLC-β_1 is detected in apical membranous portions of bronchiolar epithelium(E, ×200) and PLC-β_3 seems to localize in the nucleus of bronchiolar and alveolar epithelial cells as well as in fibroblasts(F, ×200).

small amount, was detected in the nuclear membrane and nucleoplasm of the bronchiolar and alveolar lining epithelium(Fig. 3F).

DISCUSSION

Many diverse signals are transduced through PLC in various cells and tissues. Included signals are growth factors, hormones, neurotransmitters, cytokines, inflammatory as well as the sensory signals. There are 10 known mammalian isozymes of PLC as well as couple of Drosophila and avian PLCs to provide for the diversity and specificity of the signals in combination with various cellular receptors and guanine nucleotide binding proteins which seemed to act in upstream of the PLC(Rhee and Choi, 1992).

Surveys about the distribution of PLC isozymes in animal tissues have revealed that, although some isozymes are universal, the expression and the presence of different isozymes of PLC are variable in different tissues and cell lines. For example, PLC-γ_1 and PLC-δ_1 are ubiquitously present in most tissue where as PLC-β_2 seems to be specifically found in hematologic tissues such as HL60 cells(Rhee and Choi, 1992). PLC-γ_2 appears to be important in mediating signals in B lymphocytes(Liao et al., 1993), however, in T cell receptor signaling, PLC-γ_1 and its phosphorylation plays an important role(Park et al., 1992). Established cell lines are more homogeneous in their cell population and thus contain specific isozymes of PLC in large proportions which may reflect a special function of the cells when compared to a more heterogeneous population of cells found in the tissue. For example, it is known from our experience that thyroid carcinoma cell line FRTL has high content of PLC-β_3 and -δ_4 where as GH3 cell line contain unusually high proportion of PLC-β_4, etc(unpublished data of the authors).

The elevated content and overexpressions of PLC-γ_1 has been reported in many cancers such as breast (Arteaga et al., 1991), colorectal(Noh et al., 1994), stomach(Hwang et al., 1996 ; Kim et al., unpublished) and hepatoma(Kim et al., 1994b). However, most of these studies were done before the identification of the currently known 10 mammalian PLC isozymes and they included only PLC-β_1, -γ_1, and -δ_1 isozymes of PLC in these surveys. Moreover, information regarding the distribution of PLC isozymes in human lung tissue is limited. Therefore, we thought it appropriate and timely to survey the presence of different isozymes of PLC in normal human lungs, in order to provide basic knowledge for the future studies and to understand their potential implications in disease states such as lung cancer, acute lung injuries, interstitial fibrosis, radiation pneumonitis and etc.

We have partially purified and fractionated different isozymes of PLC from normal human lung by successive choromatographies on heparin-sepharose conventional and TSK heparin 5PW HPLC columns. It is well known that PLC has a strong binding affinity for heparin resin(Rhee et al., 1989). Judging from the PLC activity assay in conventional heparin chromatography, more than 90% of the PLC activity present in crude total lung extract was recovered in the pooled PLC activity peaks. We have reasoned that the major portion of PLC is included in the fraction and the loss of PLC in this partial purification step was relatively minor when we consider the fact that we were able to exclude approximately 65% of the undesired protein from the extract.

Subsequent fractionation by heparin HPLC identified at least 4 peaks of PLC acivity present in the lung extracts. Western blot analysis showed that these 4 peaks of PLC acivity in human lungs corresponds to mainly 4 isozymes, PLC-$\beta_{1\&3}$, -γ_1, and -δ_1. The first eluted PLC peak was identified as being PLC-γ_1. It showed higher activity in PI substrate than in PIP_2 substrate, the finding typical and consistent with PLC-γ_1 enzyme as reported previously(Rhee et al., 1989). The second and also the largest PLC activity peak found was PLC-δ_1. It hydrolyzed both PI and PIP_2 substrate equally well which is consistent with the characteristics of PLC-δ_1(Lee et al., 1993). This PLC-δ_1 peak was followed shortly by an another small PLC-δ_1 peak designated by δ_1' in Fig. 2. The reason for PLC-δ_1' having two elution points on heparin HPLC is not certain. However, the same observation was made consistently in surveying PLC enzymes in our laboratory from rat kidneys and adrenal glands. It may represent an altered form of PLC-δ_1, thus having a different affinity for the heparin matrix(unpublished data of the authors). The peaks around fraction 49 to 51 were small in height and rather broad based, showing activity preference toward PIP_2 substrate in the assay. This broad peak contained PLC-β_1 and PLC-β_3 isozymes as identified by the specific antibodies. These findings are also consistent with the previous report that these two isozymes are very difficult to separate on HPLC but could be distinguished by an immunoblotting(Jhon et al., 1994).

Immunohistochemistry of the paraffin-embedded lung tissue was able to demonstrate the subcellular locali-

zation of the isozymes. PLC-γ_1, as reported to be ubiquitous in most tissues(Rhee et al., 1992), was identified to be present in the cytoplasm of bronchiolar and alveolar epithelium as well as in fibrobasts. It could have an important meaning in the pathogenesis of lung carcinoma as its overexpression and microinjection of PLC-γ_1 or its SH3 domain is known to cause transformation(Smith et al., 1989 ; Renard et al., 1992 ; Smith et al., 1994 ; Hwang et al., 1995) but its expression is down-regulated during the differentiation of U937 cells (Lee et al., 1995). This finding is also in agreement with the fact that NIH 3T3(McBride et al., 1991; Renard et al., 1992) and CCL39 cells(unpublished data of the authors) originating from lung fibroblasts express PLC -γ_1 in large amounts.

PLC-δ_1 was present in the cytoplasm of bronchiolar and alveolar epithelium agreeing with its presence in the cytosolic fraction of the cell lysates. Most of PLC-β_1 staining was noted in the apical membranous portion of the respiratory epithelium, which is consistent with its presence in membrane fraction of the lysates(Rhee and Choi, 1992). PLC-β_3 stained respiratory and alveolar epithelium and to our surprise, was located in the nucleus. There is a study concerning nuclear localization sequences present in PLC-β family and our finding of PLC-β_3 in the nucleus agrees with the data reported in previous literature(Kim et al., 1994a).

In our immunohistochemistry slides alveolar macrophages were also recognized by several PLC antibodies(data not shown). However, due to the sticky nature of alveolar macrophage cell surface, it was not possible to rule out non-specific bindings. Even though the presence of these isozymes of PLC in human lungs had been demonstrated in this study, the roles played by PLC isozymes in these specific cells is not known and calls for a further study.

In conclusion, to our experience PLC seems to be differentially expressed in various tissues, reflecting the unique functions and features of each tissue to a certain extent. Normal human lungs contain at least 4 distinct PLC isozymes, PLC-β_1, -β_3, -γ_1, and -δ_1 in the order of increasing amount found. The information obtained from this study could provide an useful basis for the future expansion of research to explain the potential derangements in signal transduction processes which may underly the pathogenesis of many pulmnary disorders in the area of acute injuries, hypersensitivity, fibro-proliferative conditions and malignancies.

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