Expression and localization of diacylglycerol kinase isozymes and enzymatic features in rat lung

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Katagiri, Yuji, Tsukasa Ito, Sachiko Saino-Saito, Yasukazu Hozumi, Akira Suwabe, Kazuhisa Otake, Makoto Sata, Hisatake Kondo, Fumio Sakane, Hideo Kanoh, Isao Kubota, and Kaoru Goto. Expression and localization of diacylglycerol kinase isozymes and enzymatic features in rat lung. Am J Physiol Lung Cell Mol Physiol 288: L1171–L1178, 2005. First published February 25, 2005; doi:10.1152/ajplung.00237.2004.—Diacylglycerol kinase (DGK) catalyzes phosphorylation of diacylglycerol to generate phosphatidic acid, and both molecules are known to serve as second messengers as well as important intermediates for the synthesis of various lipids. In this study, we investigated the spatiotemporal expression patterns of DGK isozymes together with the developmental changes of the mRNA expression and enzymatic property in rat lung. Northern blot and RT-PCR analyses showed that mRNAs for DGKα, -ε, and -ζ were detected in the lung. By immunohistochemical examination, DGKα and -ζ were shown to be coexpressed in alveolar type II cells and macrophages. Interestingly, these isozymes were localized at distinct subcellular locations, i.e., DGKα in the cytoplasm and DGKζ in the nucleus, suggesting different roles for these isozymes. In the developing lung, the expression for DGKα and -ζ was transiently elevated on embryonic day 21 (E21) to levels approximately two- to threefold higher than on postnatal day 0 (P0). On the other hand, the expression for DGKε was inversely elevated approximately twofold on P0 compared with that on E21. These unique changes in the expression pattern during the perinatal period suggest that each isozyme may play a distinct role in the adaptation of the lung to air or oxygen breathing at birth.

phosphoinositide; spatiotemporal expression patterns

THE PHOSPHOINOSITIDE (PI) cycle mediates one of the intracellular signal transduction pathways in eukaryotic cells and produces a class of second messengers that are involved in a variety of signaling cascades including cell growth, differentiation, hormonal and neurotransmitter action, and sensory perception. Triggering of the cell surface receptors, such as G protein-coupled receptors and receptor tyrosine kinases, initiates the cycle by activating phospholipase C (PLC), resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate into two second messengers, diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP3) (25). DG serves as an activator of phospholipid-dependent protein kinase C (PKC), whereas IP3 mobilizes Ca2+ from the endoplasmic reticulum (23). In addition to activating PKC, DG also targets other molecules, such as α- and β-chimaerins (having Rac-GTPase activating protein activity) (26) and guanyl nucleotide-exchange factors for Ras and Rap (16).

Diacylglycerol kinase (DGK) catalyzes phosphorylation of DG to generate phosphatidic acid (PA) and plays a major role in controlling the cellular level of DG (9, 33). Furthermore, there is accumulating evidence that PA, a product of DGK, also may serve as a second messenger that modulates the activity of several enzymes including PKC-ζ (20) and PLC-γ1 (15). Therefore, DGK occupies a central position in intracellular signal transduction through these two second messengers. DGK represents a large gene family of isozymes, and, in mammals, nine DGK isozymes have been isolated so far (9, 33). Previous studies have revealed that DGK isozymes show remarkable heterogeneity in structure, tissue expression, and enzymological property. We have so far reported the detailed cellular expression of mRNAs for the isozymes and their functional implications in the central nervous system and heart (5–8, 12, 32). These observations suggest that each isozyme has its own specific function in various biological processes.

The PI cycle has been shown in lung cells to mediate a variety of cellular signaling pathways (18, 22). In addition to the functional significance of DGK in signal transduction, it should also be mentioned that DG and PA are important intermediates for the synthesis of various lipids, such as phosphatidylyceroline (PC), phosphatidylethanolamine, and triacylglycerol, which are intimately involved in the biosynthetic pathway for surfactant in the lung (27). Therefore, we should take into consideration how DGK is involved in these apparently distinct mechanisms, i.e., surfactant synthesis and signal transduction.

To gain an insight into the functional implication of DGK in the lung, we first attempted to clarify the expression and localization pattern of DGK isozymes together with the developmental changes in their mRNA expressions and enzymatic properties in rat lung. Our results clearly show different localization of each DGK isozyme and its characteristic gene expression pattern in developing rat lung, suggesting that each DGK isozyme plays a different functional role in a different developing stage of the lung.

MATERIALS AND METHODS

Animals. This study was carried out in accordance with the Guide for Animal Experimentation, Yamagata University School of Medi-
cine and the Law (no. 105) and Notification (no. 6) of the Government (approval no. 04-004). Wistar rats of various developmental stages were used in this study, including adults (7–8 wk), neonates (0–2 days), and embryos of defined gestational stages. For gestational staging, the morning of the first day after conception was regarded as the embryonic (E) day 0 (E0), and the day of birth as the postnatal day 0 (P0). Rats were anesthetized with pentobarbital (10 mg/kg body wt) and killed by decapitation. The organs were rapidly removed.

Northern blot analysis. Total RNAs were extracted from whole rat lung at different stages of development and from adult rat brains (positive control) by acid guanidinium thiocyanate/phenol/chloroform extraction (TRIZol; GIBCO BRL, Bethesda, MD). Each of the total RNA samples (20 μg/lane) was denatured with formamide and size separated by formalin/agarose gel electrophoresis. The RNAs were transferred and fixed to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridized with the [32P]dATP-labeled probes for each rat DGK isoforms (α, β, γ, ε, ζ, and η) as previously described (5, 6, 7, 8, 14, 17). Quantitative analysis of the signals was performed using a densitometer (Atto Densitometer; Atto, Tokyo, Japan), and the values were normalized for relative amounts of 18S ribosomal RNA.

RT-PCR analysis. First-strand cDNA was synthesized from 2 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) following the manufacturer’s instructions. PCR amplification was performed with KOD-plus polymerase (Toyobo, Tokyo, Japan) using gene-specific oligonucleotide primers for rat DGK isoforms as follows: DGKα forward, 5′-GTGACTGTTGGACCTGTCGTT-3′; DGKα reverse, 5′-CAACACACGGCAGTTGGAGGCACC-3′; DGKβ forward, 5′-GGACGGCAGTGTTCGGACCTCT-3′; DGKβ reverse, 5′-GTTCCGGCAGTGGCCAAGGACATC-3′; DGKγ forward, 5′-GTTGGATCCACAGAGTCACTG-3′; DGKγ reverse, 5′-GACGGAGGTCCCTCTTCAC-3′; DGKε forward, 5′-CAAAGGCTCTGTAATGGCACC-3′; DGKε reverse, 5′-CCAAGGCATCTCAAACTGCGG-3′; DGKζ forward, 5′-CTGCCCATCAAAGGTAAGACGCT-3′; DGKζ reverse, 5′-GCTGTCTCGTGTCCTACCT-3′. PCR conditions were as follows: 95°C for 1 min; 30 cycles of 94°C for 30 s, 62°C for 30 s, 68°C for 40 s; and 68°C for 2 min. For normalization, rat GAPDH mRNA was simultaneously amplified using forward 5′-TTCAAGAGCCCTTGGCAGAAG-3′ and reverse 5′-CTACTCTCTGGAGGCCATG-3′ primers. PCR products amplified were separated by agarose gel electrophoresis, stained with ethidium bromide, and subjected to densitometric analysis as described above.

Immunohistochemistry. Excised lungs were endotracheally infused with 0.05 M Tris-oxidase complex system (Vector Laboratories) with diaminobenzidine antigen-antibody reaction were visualized by avidin-biotinylated peroxidase complex system (Vector Laboratories) or biotinylated goat anti-mouse IgG (for anti-ED-1 antibody, Molecular Probes), followed by streptavidin-Alexa 488 (dilution 1:250; Molecular Probes). Immunofluorescent image was observed under a fluorescence microscope (Leica Q550FW) and confocal laser-scanning microscope (LSMS PASCAL; Carl Zeiss, Jena, Germany) at 543 nm helium excitation and 488 nm argon excitation and processed using Adobe Photoshop.

Isolation of rat alveolar type II cells and alveolar macrophages. Alveolar type II cells were isolated from specific pathogen-free adult male Wistar rats by pancreatic elastase digestion and metrizamide density-gradient centrifugation, according to the method described by Dobbs and Mason (3). Alveolar macrophages were collected by bronchoalveolar lavage and used without further purification. These procedures yielded >98% purity for both cell types, which was determined by immunohistochemical staining for anti-SP-C or ED-1 antibody (data not shown). Isolated cells were placed on glass microscope slides by cytopsin preparation and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, and immunohistochemical staining was performed as described above. Some of the cells were used for enzymatic assay for DGK.

Protein extraction and DGK activity. For measurement of DGK activity, total protein was extracted from adult rat brain, lung, isolated alveolar type II cells, and alveolar macrophages using lysis buffer. The homogenates were centrifuged at 14,000 g for 10 min to remove cell debris. Resulting supernatants were used for the assay. The protein concentration was determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). DGK activity was measured by octylglucose-mixed micelle assay using 1-stearyl-2-linoleoyl-sn-glycerol (C18:0/C18:2 DG) (Biomol, Plymouth Meeting, PA) and 1-stearyl-2-arachidonoyl-sn-glycerol (C18:0/C20:4 DG) (Biomol, Plymouth Meeting, PA) as previously described (10). Assay was performed in the presence of each substrate at a concentration of 1 mM. The reaction mixture (50 µl) contained 50 mM MOPS (pH 7.2), 50 mM octylglucoside (Calbiochem, San Diego, CA), 100 mM NaCl, 1 mM dithiothreitol, 20 mM NaF, 2.1 mM CaCl2, 2.0 mM EGTA, 10 mM biochem, San Diego, CA), 100 mM NaCl, 1 mM dithiothreitol, 10 mM MgCl2, 6.7 mM phosphate buffer (Avanti Polar Lipids, Birmingham, AL), and 1 mM [γ-32P]ATP (10,000 counts per minute/nmol; ICN Biomedicals, Costa Mesa, CA). The reaction was continued for 10 min at 30°C. Lipids were extracted and separated on thin-layer plates of silica gel (Merck, Darmstadt, Germany). The band of PA detected by autoradiography was scraped with a sharp spatula and collected for liquid scintillation counting. Under the conditions described above, the rate of the reaction was linear for at least 10 min.

Statistics. Values are shown as means ± SD for each experimental group. Groups were compared using one-way analysis of variance and the Tukey-Kramer multiple-comparison tests for differences between groups. P < 0.05 was considered to be statistically significant.

RESULTS

Expression and localization of DGK isoforms in the lung. In Northern blot analysis, hybridization signals for the three DGK isoforms (α, ε, ζ) were detected in adult rat lung (Fig. 1A). Positions of the hybridization bands for the three isoforms were the same as those in the brain. The expression levels based on the strength of the hybridization bands of DGKα and -ζ were high, whereas that of DGKε was low. RT-PCR analysis also confirmed the expression profile of the DGK isoforms in the lung described above (Fig. 1B).

To examine which cells were responsible for the expression, we performed immunohistochemical analysis of the lung using specific antibodies against rat DGKα and -ζ (Figure 2). Immu-
Expression of DGKα and -ζ in isolated alveolar type II cells and macrophages. We further examined the expression of DGKα and -ζ using isolated alveolar type II cells and macrophages. The isolation procedures yielded ~98% purity for both cell types, which was determined by immunocytochemical staining for anti-SP-C or anti-ED-1 antibody (data not shown). Immunoreactivities for DGKα and -ζ were clearly detected in both cell types, with the former fine dotted in the cytoplasm and the latter speckled in the nucleus (Fig. 5). These results were the same as those of the immunostaining in the lung, further confirming that DGKα and -ζ are expressed in alveolar type II cells and macrophages.

DGK activity in the whole lung, alveolar type II cells, and macrophages. In the previous study, DGK activity has been detected in rat lung (13). However, recent molecular cloning studies of several DGK isoforms have revealed different enzymological properties of the isoforms, i.e., DGKα, -β, and -γ are activated by Ca2+, and DGKε specifically phosphorylates arachidonoyl-containing DG (1-stearoyl-2-arachidonoyl-sn-glycerol) (33). Therefore, we reexamined DGK activity in the whole lung with attention to Ca2+ dependency and substrate specificity. We also examined the enzymatic activity of the isolated alveolar type II cells and macrophages. An octyl-glucoside-mixed micelle assay was used to evaluate substrate specificity toward 1-stearoyl-2-linoleoyl-sn-glycerol (18:0/18:2 DG) and 1-stearoyl-2-arachidonoyl-sn-glycerol (18:0/20:4 DG) in the presence of 0.1 mM Ca2+ (21). As shown in Fig. 6A, DGK activity was detected in the whole lung at 10-fold lower levels in the brain where most of the isoforms are abundantly expressed. The activities toward 18:0/18:2 DG and 18:0/20:4 DG were almost comparable in the lung, showing no apparent substrate specificity. DGK activities in the isolated alveolar type II cells and macrophages were ~20% and 150% compared with that of the whole lung, respectively, and almost equal toward 18:0/18:2 DG and 18:0/20:4 DG in the presence (0.1 mM) or absence of Ca2+, the whole lung and the isolated cells showed similar enzymatic activities (Fig. 6B).

Double-immunofluorescent staining of alveolar type II cells and macrophages. To confirm the expression of DGKα and -ζ in alveolar type II cells and macrophages, we performed double-immunofluorescent analysis using specific markers for those cells, i.e., anti-SP-C antibody and anti-ED-1 antibody for alveolar type II cells and macrophages, respectively. As shown in Fig. 3A, most of the DGKα-immunoreactive cells were also SP-C immunoreactive, indicating that DGKα is expressed in alveolar type II cells. On the other hand, DGKζ-immunoreactive cells outnumbered SP-C-immunoreactive cells in the alveoli (Fig. 3B), suggesting that DGKζ is expressed not only in type II cells but also in type I cells, although at low levels. With regard to alveolar macrophages, ED-1-immunoreactive macrophages were also immunoreactive for both DGKα and -ζ, showing that these isozymes are expressed in these cells (Fig. 4).

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Expression of DGK isozymes in the developing lung. Development of the lung begins on approximately E11 in rats as a ventral foregut outpouching. Subsequently, repetitive branching of the lung bud occurs with eventual formation and maturation of the alveolar acinar units, together with specialized epithelial cell differentiation and surfactant production. In the perinatal period, the sudden exposure of the air-blood interface of the lung to increased oxygen tension must cause drastic changes to lung cells. To understand the functional implications of DGK isozymes during lung development, we examined temporal expression patterns of mRNA for DGK isozymes in the developing lung by Northern blot analysis using 18S ribosomal RNA as a control (Fig. 7A). Hybridization signals for DGKα showed a constant level during the embryonic stage but gradually increased after birth. At adult stage, the signals were approximately fourfold higher than those of the embryonic stage and almost equivalent to those of adult brain. On the other hand, the hybridization signals for DGKε were nearly constant during embryonic and postnatal stages. A similar expression pattern was found in DGKζ except that the signals increased slightly after birth.

It should be noted, however, that during the perinatal period, distinct expression patterns were observed for DGKα, -ε, and -ζ (Fig. 7A): the expression of DGKε was transiently elevated immediately after birth (P0) compared with that on E21. On the other hand, the expression for DGKα and -ζ was inversely elevated on E21 and then decreased on P0. To further examine these changes during the perinatal period, we used semiquantitative RT-PCR with GAPDH as a control (Fig. 7B). The data clearly showed that the expression for DGKε was transiently elevated approximately fourfold on P0 compared with that on E21 and then decreased on P1, although changes in the expression for DGKα and -ζ during the perinatal period were not statistically significant. These data suggest that DGK isozymes play distinct roles in developing lung, especially during the perinatal period.

Fig. 3. Immunofluorescent localization of DGK isozymes and surfactant protein (SP)-C in the alveolus. Adult rats were fixed and double immunostained as described in MATERIALS AND METHODS. Images of the 2 fluorophores were colorized and combined (merge). Top: low-magnified images under the fluorescent microscopy; bottom: images of representative cells under confocal laser scanning microscopy. A: immunofluorescent localization of DGKα and SP-C (a marker for alveolar type II cells). Red indicates Alexa 546 staining of DGKα, and green indicates Alexa 488 staining of SP-C. DGKα immunoreactivity is detected in the cytoplasm of round alveolar cells located near the angles between neighboring septa (arrowheads), where it colocalizes with SP-C immunoreactivity in one and the same cells. B: immunofluorescent localization of DGKζ and SP-C. Red indicates Alexa 546 staining of DGKζ, and green indicates Alexa 488 staining of SP-C. DGKζ immunoreactivity is detected intensely in the nucleus of the round alveolar cells (arrowheads), where it colocalizes with SP-C immunoreactivity in one and the same cells. In addition, DGKζ immunoreactivity is also detected moderately in the nucleus of squamous alveolar cells that are not immunoreactive for SP-C (arrows). Bar = 10 μm.

Fig. 4. Immunofluorescent localization of DGK isozymes and ED-1 in the alveolus. Adult rats were fixed and double immunostained as described in MATERIALS AND METHODS. Images of the 2 fluorophores were colorized and combined (merge) under confocal laser scanning microscopy. A: immunofluorescent localization of DGKα and ED-1 (a marker for macrophages). Red indicates Alexa 546 staining of DGKα, and green indicates Alexa 488 staining of ED-1. DGKα immunoreactivity is detected in the cytoplasm of round alveolar cells located in the alveolar space, where it colocalizes with ED-1 immunoreactivity in one and the same cells. B: immunofluorescent localization of DGKζ and ED-1. Red indicates Alexa 546 staining of DGKζ, and green indicates Alexa 488 staining of ED-1. DGKζ immunoreactivity is detected in the nucleus of free cell in the alveolus, where it colocalizes with ED-1 immunoreactivity in one and the same cells. Bar = 10 μm.
that propagates the IL-2-mediated signal transduction may be shared among relevant cells in general, the expression of DGKξ in these lung cells, as revealed in this study, suggests that this isozyme might be involved in this pathological process in the lung, which warrants further investigation.

Nuclear localization of DGKξ in the lung cells is compatible with our previous study of its immunohistochemical examination in neurons (12), which together with this study, indicates that DGKξ is localized in the nuclei of both proliferating and nonproliferating cells. Although the functional role applicable to all kinds of DGKξ-expressing cells is unclear, our previous study of myocardial infarction model of rats shows that the mRNA expression for DGKξ is enhanced in macrophages that are infiltrated into the necrotic area, suggesting the possible role of DGKξ in phagocytosis of these cells (32). It remains to be elucidated whether macrophages intrinsically express DGKξ at a high level or whether the increased expression is induced by the activation of the phagocytic reaction.

One of the best-characterized functional roles of DGK is in the regulation of PKC, for which DG acts as an allosteric activator (23). In this regard, there is evidence that PKC may...
play an important role in the regulation of surfactant secretion in type II cells (28, 29). Agonists that activate PKC, such as 12-0-tetradecanoylphorbol-13-acetate and cell-permeable DGs, are shown to serve as the most effective surfactant secretagogues in isolated type II cells (29). Rooney et al. (28) describes that ATP triggers the PI cycle via P2Y2 purinoceptors, which results in the activation of PKC and subsequent stimulation of surfactant secretion. Furthermore, calphostin C, a potent inhibitor of PKC, blocked PC secretion stimulated by glucagon-like peptide 1, the truncated and amidated form of glucagon-like peptide 1, in human alveolar type II cells (34). Thus it is highly plausible that DGK may play a role in regulating surfactant secretion, although which one is responsible for the regulation among the three isoforms identified in this study remains to be elucidated.

Enzymatic assays for the whole lung, isolated alveolar type II cells, and macrophages reveal that total DGK activities in these samples show neither Ca²⁺ dependency nor substrate specificity. To date, enzymatic property has been well characterized for DGK isozymes: Ca²⁺/H₁₁₀₀₁-dependent activation for DGKα, -β, and -γ, specific phosphorylation toward arachidonoyl-containing DG for DGKε (33). Considering the present Northern blot and RT-PCR data showing that DGKα, -γ, and -ε are responsible for the expression in the lung, DGKγ, a Ca²⁺-independent isozyme with no substrate specificity, may be dominant among those in terms of the activity.
It should also be mentioned that DGK isozymes show unique patterns of expression during the perinatal period. Using semiquantitative RT-PCR, we found that the expression for DGKε was transiently elevated at approximately fourfold immediately after birth (P0) during the perinatal period. Lung is one of the organs exposed to drastic changes before and after birth. In this regard, the biochemical adaptation of the lung to air or oxygen breathing at birth is incompletely understood. A sudden exposure of the air-blood interface of the lung to increased oxygen tension must pose an acute oxidative stress compared with the relatively anaerobic fetal environment. Indeed, the pulmonary epithelium is usually exposed to the highest oxygen tension present in the organism (2). Our data show that DGKε is very unique in that its expression is upregulated by increased oxygen tension, whereas expression for DGKα and -ζ is downregulated. It is known that PI has a characteristic fatty acid composition of 1-stearoyl-2-arachidonoyl (11). Considering the substrate specificity of DGK towards arachidonoyl-DG, a possible link might be suggested between oxygen stress and PI metabolism involved with DGKε. This hypothesis may be partly consistent with the previous report that reactive oxygen species, such as hydrogen peroxide, activate several enzymes involved in lipid signaling, such as PI-specific PLC, in several cultured cell types (31).

Further studies are needed to clarify this point.

In conclusion, our results reveal for the first time the gene expression of DGKα, -ζ, and -ε in the lung. Furthermore, immunohistochemical analysis shows that DGKα and -ζ are coexpressed in alveolar type II cells and macrophages and that these isozymes are localized at distinct subcellular locations. In the developing lung, distinct expression patterns for DGKα, -ε, and -ζ are observed during the perinatal period. All these data suggest that each isozyme plays a different role in lung functions such as surfactant production and secretion, phagocytic reaction, and adaptation to oxygen breathing. Identification of specific isozymes of DGK in the lung would help us further investigate detailed physiological and pathological roles for each molecule.

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