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Localization of Diacylglycerol Kinase ζ in the Kidney of Adult Rats: Its Dominant Expression in Collecting Tubules and its Selective Lack in Proximal Tubules

Localización de Diacilglicerol Quinasa ζ en el Riñón de Ratas Adultas: su Expresión Dominante en Túbulos Colectores y su Carencia Selectiva en Túbulos Proximales

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SUMMARY: Diacylglycerol kinase (DGK) exerts balancing the intracellular level between two-second messengers, diacylglycerol and phosphatidic acid, by its phosphorylation activity. DGK ζ is often localized in cell nuclei, suggesting its involvement in the regulation of intranuclear activities, including mitosis and apoptosis. The present immunohistochemical study of rat kidneys first revealed no detection levels of DGK ζ -immunoreactivity in nuclei of most proximal tubule epithelia in contrast to its distinct occurrence in cell nuclei of collecting and distal tubules with the former more dominant. This finding suggests that DGK ζ is a key factor regulating vulnerability to acute kidney injury in various renal tubules: its low expression represents the high vulnerability of proximal tubule cells, and its distinct expression does the resistance of collecting and distal tubule cells. In addition, this isozyme was more or less localized in nuclei of cells forming glomeruli as well as in endothelial nuclei of peritubular capillaries and other intrarenal blood vessels, and epithelial nuclei of glomerular capsules (Bowman's capsules) and renal calyces, including intrarenal interstitial cells.

KEY WORDS: DGK ζ; Kidney; Rat; Nucleus; Renal tubule.

INTRODUCTION

In the phosphoinositide (PI) cycle, diacylglycerol (DAG) and phosphatidic acid (PA), though minor among the intramembranous phospholipids, carry out specific tasks for a wide range of biological processes in eukaryotic cells. DAG regulates activities of conventional PKC (cPKC), novel PKC (nPKC), protein kinase D, chimaerin (Rac-specific GTPase-activating protein (GAP)) and Ras guanyl nucleotide-releasing protein (GRP). Moreover, the transient receptor potential channel TRPC2 (Ca^{2+}-permeable cation channel) is gated by DAG (Ohno & Nishizuka, 2002). On the other hand, PA has also been reported to regulate a number of signaling proteins such as phosphatidylinositol (PI)-4-phosphate 5-kinase (PIP5K), RasGAP, Raf-1 kinase, mammalian target of rapamycin (mTOR), and atypical PKC. chimaerin is activated by PA as well (Goto *et al*., 2007). The cellular concentrations of such important bioactive lipids, DAG and PA, must be strictly regulated by the action of their metabolic enzymes. Because DAG kinase (DGK) phosphorylates DAG to generate PA, this enzyme can potentially regulate the balance between DAG and PA by serving as a DAG consumer as well as a PA generator. Despite such a high variety of functional roles of DGK as well as these two PIs which are likely to be essential in the kidney function considering their specific roles described above, information on detailed localization of DGK in various cells comprising the kidney *in vitro* and *in situ* is insufficient, especially little in renal tubule cells *in situ* (Troyer *et al*., 1986; Staiano & De Matteis, 2019).

Considering these issues, and on the basis of a series of our studies on the localization of various isozymes of PI signal-implicated enzymes in various peripheral organs as

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well as the brain (Nakamura *et al*., 2004; Nomura *et al*., 2007; Goto *et al*., 2007; Nakano *et al*., 2012; Hozumi & Goto, 2012; Hipkaeo *et al*., 2015; Khrongyut *et al*., 2019; Chomphoo *et al*., 2021, Pakkarato *et al*, 2022), we have recently clarified that DGK z is confined to microvilli of the proximal convoluted tubule cells in the cortex and partially to those of the proximal straight tubule cells in the medullary ray (Hemha *et al*., 2023). As the second of our analysis on the localization of DGK in the kidney, the present study attempted to analyze the localization of DGK z in the kidney of adult rats.

MATERIAL AND METHOD

Animal preparation. Eight-week-old Sprague Dawley rats were purchased from the Siam Nomura Laboratory Animal Center, Co., Ltd. Bangkok, Thailand. Rats were fed ad libitum and kept at 23-25 ºC at 12h/12h dark/light cycles. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Khon Kaen University and approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethical Principles and Guidelines for the Use of Animals: National Research Council of Thailand (reference No. IACUC-KKU-25/63).

Kidney sample collection. Under anesthesia with thiopental sodium (Cat# JPN3010001, Jagsonpal Pharmaceuticals, Hayana, India; 100 mg/kg body weight), all the rats were transcardially perfused with 100 ml physiological saline, followed by 100 ml 4 % paraformaldehyde/0.1 M phosphate buffer (PB). Kidneys were removed, and post-fixed with the same fixative overnight. The specimens were then dipped into 30 % sucrose/0.1M PB for cryoprotection until used for immunohistochemical analysis.

Immunohistochemistry in Light and Electron Microscopy. Cryosections (15 μm thickness) of kidney samples were prepared and put on glass slides. Intrinsic peroxidase activity was inhibited by incubation with 0.3 % H_2O_2/methanol for 10 min. Non-specific antibody binding was prevented by treatment of tissue sections with 10 % normal goat serum/PBS for 30 min. The sections were then incubated overnight at 4 °C with the same polyclonal antibody against rat DGK ζ as that used in our recent study (Hemha *et al*, 2023) at the final concentration of 1 μg/ml in 0.1 % Triton-X/PBS. The specificity of the antibody and immunoblot data of homogenates of rat kidneys were already described in that study. The sections were subsequently incubated for 1 h at room temperature with biotinylated anti-rabbit IgG secondary antibody (Cat# ab64256, RRID: AB_2661852, Abcam, Cambridge, MA, USA) for the DAB reaction using an ABC kit (Cat# PK-6100, RRID: AB_2336819, Vector Laboratories, Burlingame, CA, USA), and observed using a light microscope (Leica DMRXE, connected with DMC2900 camera, Leica Microsystems GmbH).

For identification of individual renal ducts immunoreactive for DGK ζ several pairs of two consecutive cryostat sections were made, and each of the sections mounted on two different glass slides was incubated with either immuno-markers for individual tubules and DGK ζ-antibody in such a way as a section-glass with DGK ζ-antibody sandwiched by the other two section/glasses with one or two immuno-markers. An antibody for aquaporin 1 (AQP1, Cat# AB 2219, RRID: AB_1163380, Merck Millipore, Billerica, MA, USA) was used as a marker of the proximal tubules, and antibody for aquaporin 2 (AQP2, Cat# 178612, RRID:AB_10685039, Merck Millipore, Billerica, MA, USA) was as a marker of the connecting tubules. Because all the antibodies used in this study were generated from rabbit IgG, this immunostaining method using consecutive sections was the only way for the examinations, but the double immunofluorescence method on confocal laser scanning microscopy was not applicable, unfortunately. However, as far as given uriniferous tubules were contained in both of consecutive sections, the examination of co-localization could be performed reliably as shown in results below.

For immuno-DAB-electron microscopy, the immunostained kidney sections were osmicated with 0.5 % OsO4/0.1M phosphate buffer (PB) for 30 min, and subsequently treated in bloc with 0.5 % uranyl acetate for 30 min. They were then dehydrated with 70 %, 80 %, 90 % and 95 % of ethanol for 5 min each, and finally with absolute ethanol 3 times for 15 min each, and subsequently embedded in Epon embedding Kit (EMbed 812; Cat#14120, EMS, Hatfield, PA, USA). Ultrathin sections were made and observed using a transmission electron microscope (JEM1010, Jeol, Tokyo, Japan, which was connected with a XR51 TEM imaging system, version 700, AMT, MA, USA).

As a control for the immunohistochemistry work, sections were treated with non-immune serum.

RESULTS

In immuno-light microscopy of the kidney of normal rats, DGK ζ-immunoreactivity was seen weakly to moderately in nuclei of numerous epithelia of renal tubules,

epithelial cells of glomerular capsules (Bowman's capsules), cells comprising glomeruli, endothelia of peritubular capillaries, arterioles, and venules. It was also seen in nuclei of intrarenal interstitial cells and epithelial cells of the renal calyces. However, DGK ζ-immunoreactivity was at negligible levels in cell nuclei of renal tubules whose epithelia were relatively thick with brush margins being well-developed. Such tubules with negligible immunoreactivity comprised a considerable population of total renal tubules in the cortex (Figs. 1a, 3a, 4a). In contrast, DGK ζ-immunoreactivity appeared much more distinct in nuclei of renal tubules which were relatively sparse, and the tubules often appeared in a tangentially sectioned view in the cortex and medulla. The distinctly immunoreactive tubules were differentiated into two types: One was composed of cells that were shorter in cell height and weak immunoreactivity

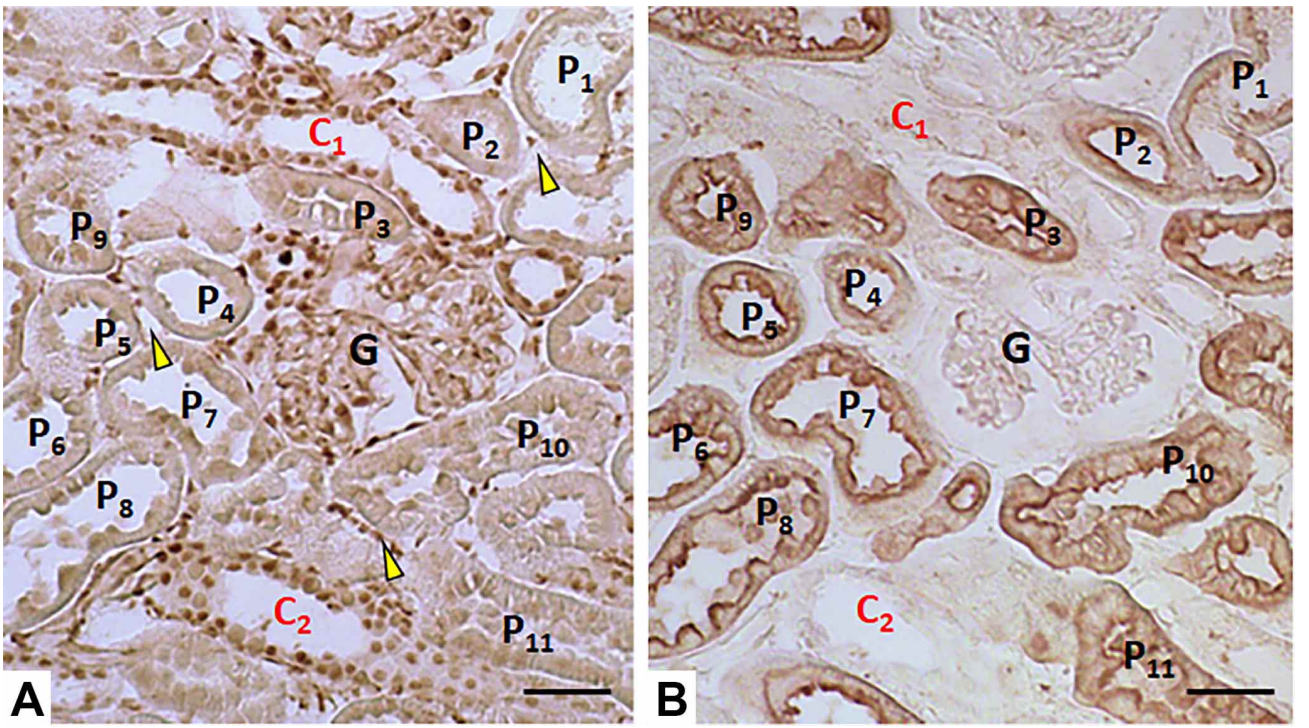

Fig. 1. A set of two consecutive cryostat sections of renal cortex immunostained alternatively for DGK ζ (a) and AQP1 (b: proximal tubule marker). Tubules corresponding to each other in (a) and (b) are labeled with C or P. The same tubules are marked in the same pair numbers. Longitudinally sectioned profiles of tubules (C, finally identified as the collecting tubules in text) show distinct nuclear DGK ζ-immunoreactivity and weak cytoplasmic immunoreactivity. Note that proximal tubules (P) identified by AQP1-immunoreactivity are almost DGK ζ-immunonegative and discrete from collecting tubules. Arrowheads indicate DGK ζ-immunoreactive nuclei of peritubular capillary endothelia and interstitial cells. G: glomeruli. Bars represent 50 μm

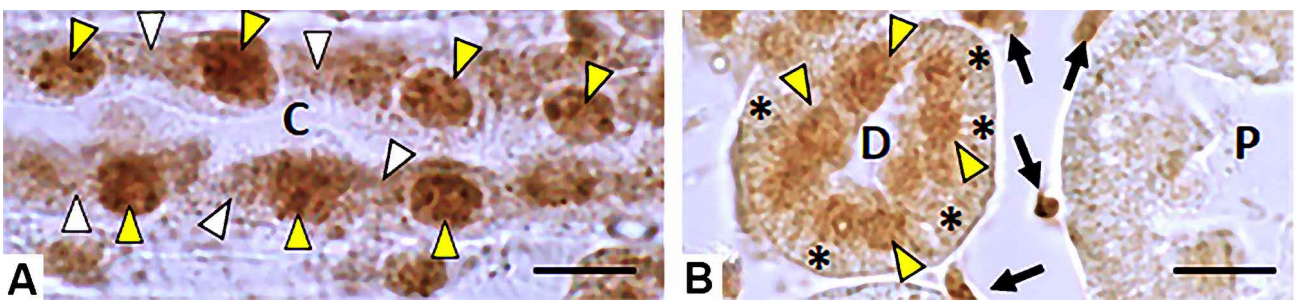

Fig. 2. Higher magnification immuno-light micrographs show a longitudinally sectioned collecting tubule (a), and cross-sectioned distal tubule and proximal tubule (b) in the cortex. Note weak cytoplasmic DGK ζ-immunoreactivity (white arrowheads) as well as distinct nuclear immunoreactivity (yellow arrowheads) in the collecting tubule (C) (a). Also, note immunonegative basal striations (*) of the distal tubule (D) having immunoreactive nuclei (yellow arrowheads) and a relatively smooth lumen (b). The proximal tubule (P) with apical brush margins showed negligible levels of immunoreactivity in nuclei or cytoplasm (b). Black arrows indicate DGK ζ-immunoreactive nuclei of likely interstitial or endothelial cells in peritubular spaces. Bars represent 10 μm

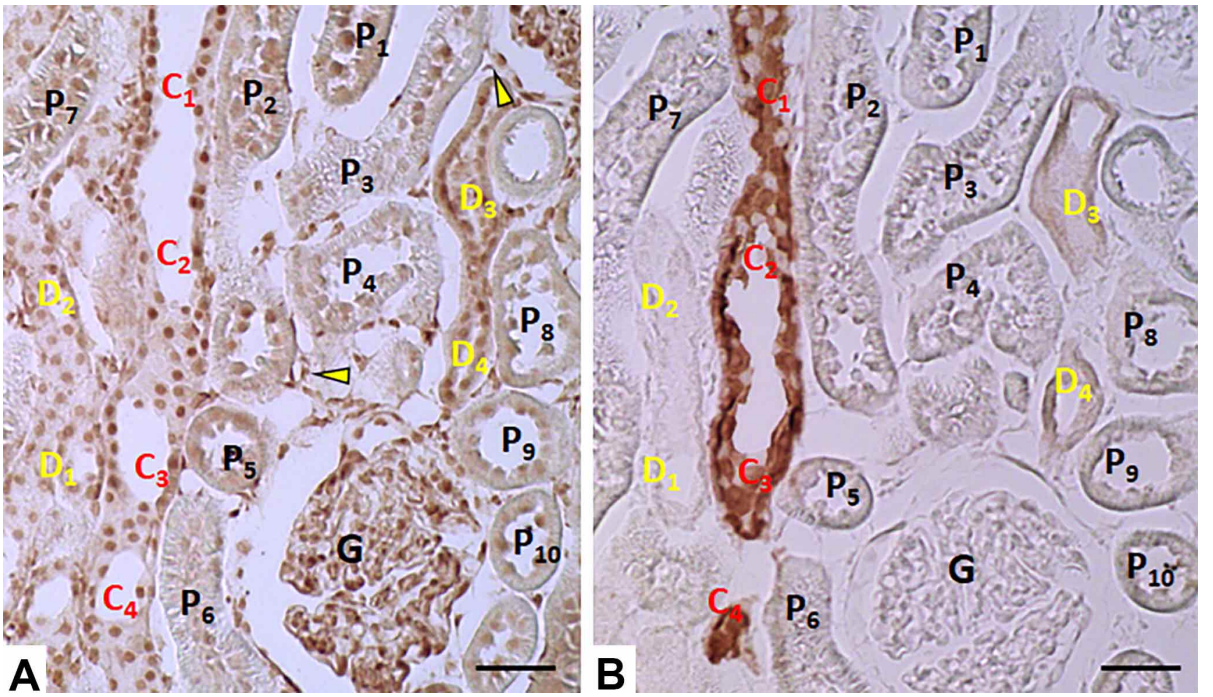

Fig. 3. A set of two consecutive cryostat sections of renal cortex immunostained alternatively for DGK ζ (a) and AQP2 (b: collecting tubule marker). The same tubules are marked in the same pair numbers. Note that tubules with distinct nuclear DGK ζ -immunoreactivity are collecting tubules (C) identified with AQP2-immunoreactivity. Also, note the presence of tubules (D) with distinct nuclear DGK ζ -immunoreactivity, which are AQP2-immunonegative. They are likely to be distal tubules. P: proximal tubules without DGKζ-immunoreactive nuclei. G: glomeruli. Bars represent 50 μm

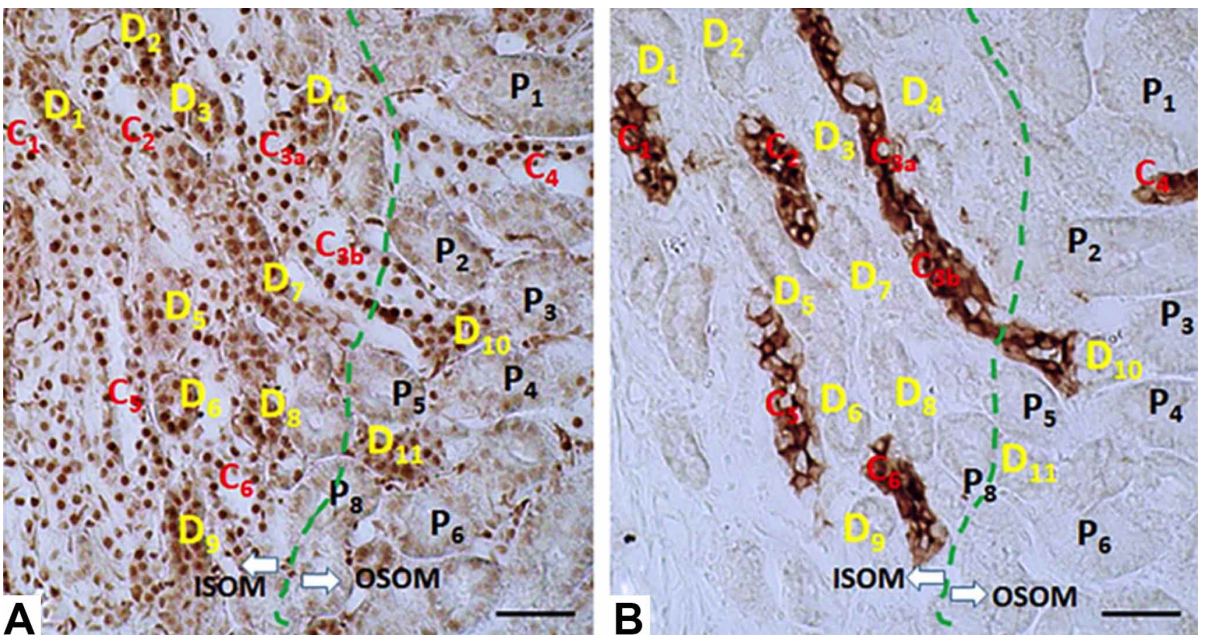

Fig. 4. A set of two consecutive cryostat sections immunostained alternatively for DGK ζ (a) and AQP2 (b: collecting tubule marker) in an area including both the outer stripe of the outer medulla (OSOM) and the inner stripe of the outer medulla (ISOM), whose boundary is indicated with green broken lines. Tubules marked with C, D, and P numbers in (a) and (b) correspond to each other. Note that AQP2-immunoreactive collecting tubules exhibit distinct nuclear DGK ζ -immunoreactivity. Also, note that tubules (D) with distinct nuclear DGK ζ -immunoreactivity in ISOM are not AQP2-immunoreactive collecting tubules. They are regarded as thick ascending limbs (TAL, straight distal tubules) because thick tubules in ISOM are known to be collecting and TAL. P: proximal tubules with DGK ζ -immunonegative nuclei. Bars represent 50 μm

was additionally discerned in most of their cytoplasm at higher magnification. The other was composed of cells that were taller in cell height and had the basal striation domain without immunoreactivity (Figs. 2a, b). The immunoreactivity within their nuclei, especially of the former cells, was recognized as accumulations of numerous tiny granules (Fig. 2a, Fig. 7 inset).

In sets of two consecutive cryostat sections which were immunostained alternatively for DGK ζ and AQP1, or for DGK ζ and AQP2, almost all epithelial cells of renal tubules immunopositive for AQP1 corresponded to tubules with negligible levels of nuclear DGK ζ-immunoreactivity in the cortex. On the other hand, almost all renal tubules immunopositive for AQP2 corresponded to tubules composed of cells having distinctly DGK ζ-immunopositive nuclei and a shorter cell height in the cortex and medulla (Figs. 1a,b; 3a,b). However, a substantial number of tubules with distinctly DGK ζ-immunoreactive nuclei were present as those not corresponding to AQP2-immunoreactive tubules in the cortex and outer medulla, and they were composed of cells having a taller cell height. Especially in the inner stripe of the outer medulla, noticed was the presence of tubules having distinct DGK ζ-immunoreactive nuclei without immunoreactivity for AQP2 (Figs. 4 a,b). In the inner medulla, AQP2-immunoreactive tubules with a short cell height of epithelia were distinctly DGK ζ-immunoreactive in nuclei and weakly immunopositive in their cytoplasm. On the other hand, nuclei of thin tubules immunoreactive for AQP1 (likely descending Henle loop) were also DGK ζ -immunoreactive, though weaker than those of AQP2-immunoreactive tubules (Figs. 5a,b; 6a,b).

Immuno-electron microscopy, granular DGK ζ -immunoreactive materials were dispersed in cell nuclei of two types of tubules: One was characterized by a shorter cell height, poorly developed apical microvilli and basal infoldings, and the other was characterized by a taller cell height, well-developed basal infoldings, but poorly developed microvilli in the cortex and the inner stripe of the outer medulla (Figs. 7a, b, inset). Immunoreactive materials were sparsely discerned in the cytoplasm in the former. No immunoreactive materials were found in the nuclei of tubule cells characterized by a taller cell height, well-developed microvilli, and basal infoldings in the cortex (data not shown).

In control experiments with non-immune serum instead of the specific individual antibodies, no significant immunoreactivities were discerned across sections of kidneys of adult rats in the immuno-LM study (data not shown).

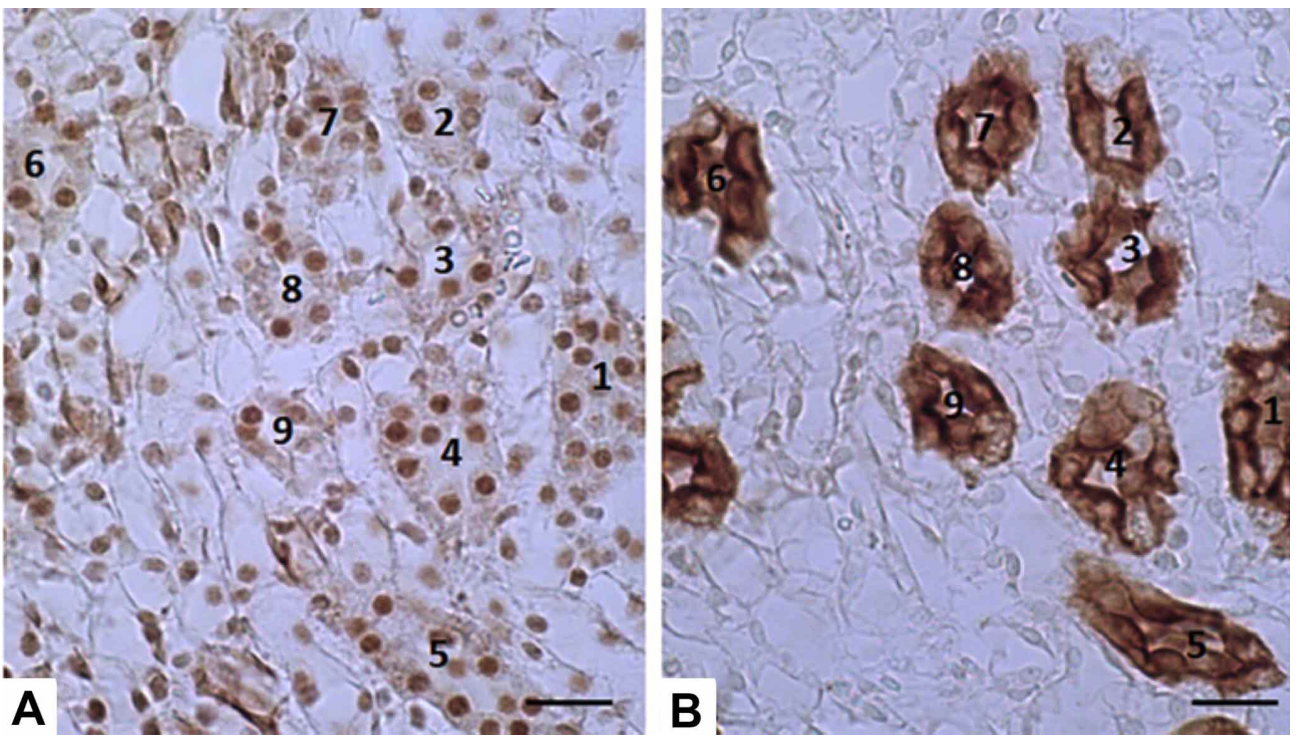

Fig. 5. A set of two consecutive cryostat sections immunostained alternatively for DGK ζ (a) and AQP2 (b) in the inner medulla. Note that collecting tubules identified with AQP2 correspond to those characterized by a substantial height of cells, distinctly DGK ζ-immunoreactive nuclei, and weakly immunoreactive cytoplasm. Corresponding tubule profiles in (a) and (b) are labeled with the same numbers. Bars represent 50 μm

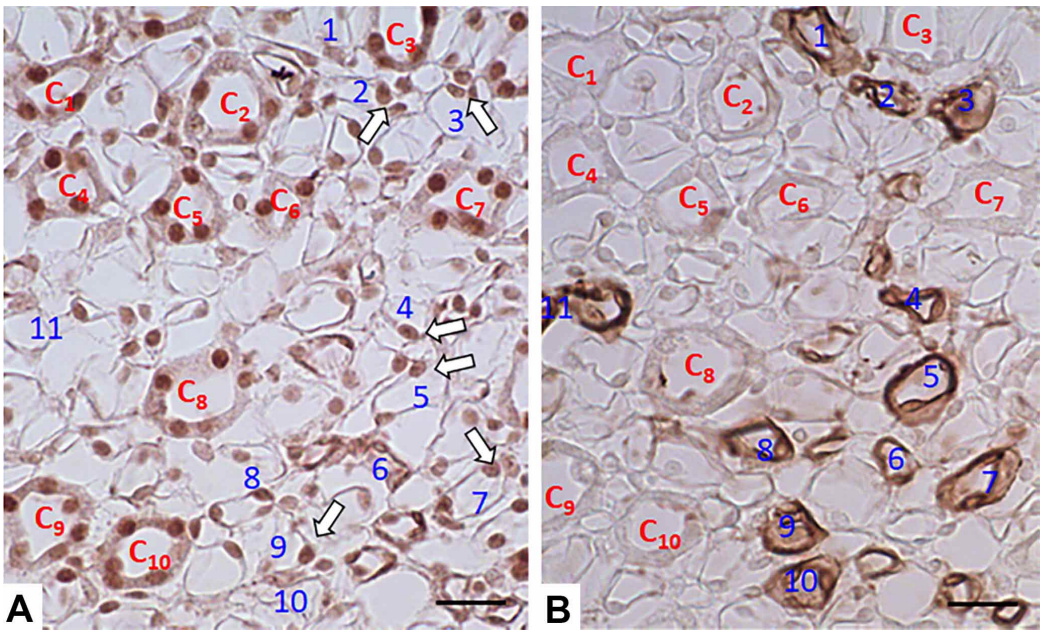

Fig. 6. A set of two consecutive cryostat sections immunostained alternatively for DGK ζ (a) and AQP1 (b) in the inner medulla. Note that nuclei (thick arrows) of descending thin limbs immunoreactive for AQP1 (1-11) are DGK ζ -immunoreactive, though weaker than those of collecting tubules (C1-C10) characterized by a substantial height of epithelia, distinct nuclear DGK ζ -immunoreactivity and weak cytoplasmic one. Corresponding tubule profiles in (a) and (b) are labeled with the same numbers. Bars represent 50 µm

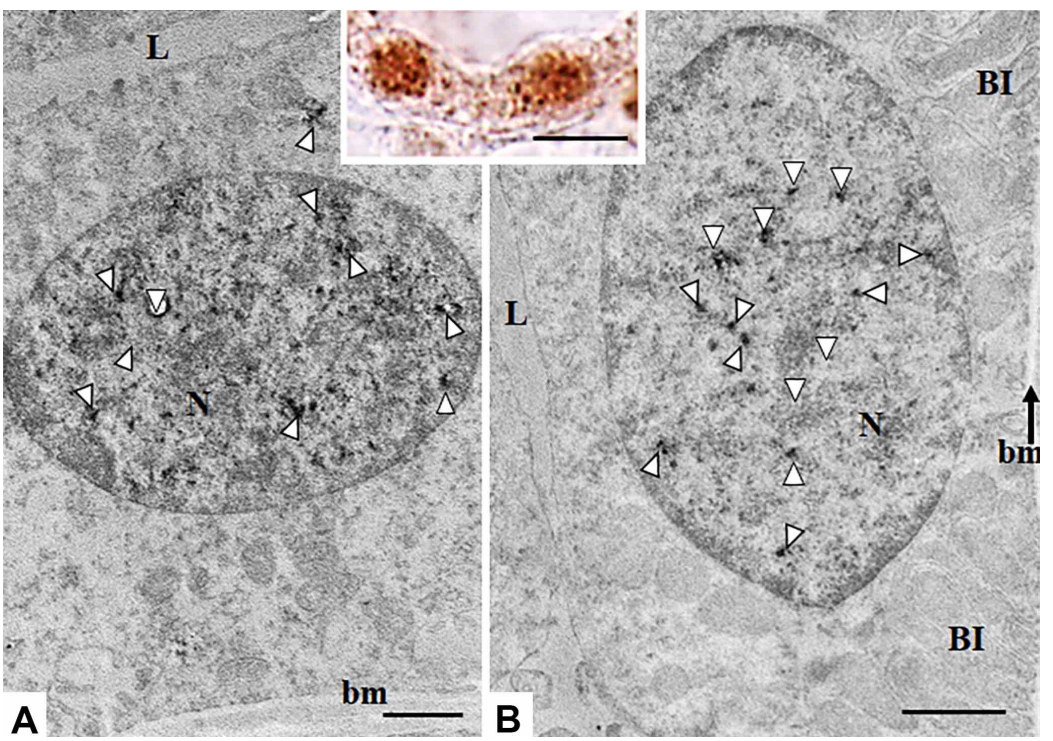

Fig. 7. Immunoelectron micrographs of collecting tubule (a) in the inner stripe of the outer medulla, and distal tubule (b) in the cortex. Intranuclear DGk ζ -immunoreactive materials appear in the forms of electron-dense dots (arrowheads) in collecting and distal tubule cells with a pattern corresponding to that in immuno-light microscopy as shown in inset and Fig 2a. Sparse immunoreactive dots are indicated by an arrowhead in the cytoplasm of collecting tubule cell. BI: basal infolding, bm: basement membrane, L: tubule lumen, N: nucleus. Bars represent 1µm (a), 500 nm (b), and 10 µm (inset).

Finally, in a preliminary observation of the kidneys from adult rats received a daily injection of gentamycin for 1 week which were used in our recent study to examine some expression changes of other PI signal-implicated molecules (Hemha *et al*., 2023), no significant changes were noticed in localization and intensity of DGK ζ -immunoreactivity in any renal cells of kidneys when compared with the present findings (data not shown).

DISCUSSION

In our recent study (Hemha *et al*., 2023), two closely located bands for DGK ζ, whose sizes well corresponded to its established band size in the brain (104 kDa), were reported to occur in western blots of kidney homogenates by using the same antibody as employed in the present study. Since several alternative splicing products of DGK ζ have been found by one (KG) of the present authors (Goto *et al*., 2007), the occurrence of such two closely located immuno-bands is acceptable for consideration that immunoreactivities for DGK ζ in the present immunohistochemistry model represent the authentic molecule.

From the present immunohistochemical finding by using two consecutive sections immunostained alternatively for DGK ζ and AQP1 or DGK z and AQP2, it is reasonable to identify tubules composed of cells exhibiting negligible levels of DGK ζ-immunoreactivity as the proximal tubules, and those having distinct nuclear DGK ζ-immunoreactivity together with a shorter cell height as the collecting tubules. This identification was confirmed by immuno-DAB-electron microscopy individually showing characteristic ultrastructural features of the two tubules.

About the identification of tubules composed of cells having distinct nuclear DGK ζ -immunoreactivity and a taller cell height, noticeable is a lack of AQP2-immunoreactivity in these tubules in the inner stripe of the outer medulla. Since the inner stripe contains only thick ascending limbs (TALs, thick distal tubules) and the collecting tubules as relatively thick renal tubules, it is reasonable to regard the tubules under discussion as TALs. This is further supported by the present immuno-electron microscopy showing the presence of nuclear immunoreactions in tubule cells having basal infoldings and less-developed apical microvilli in the inner stripe of the outer medulla as well as the cortex. It is appropriate to further confirm that tubules with distinct DGK ζ -immunoreactivity solely in nuclei and thus a relatively bright appearance are the distal tubules in the cortex, by using a reliable immunohistochemical marker for the distal tubules.

Consequently, a major finding in the present study is a selective lack of DGK ζ -immunoreactivity in cell nuclei of the proximal tubules in the cortex and the outer stripe of the outer medulla. An additional major finding is a prominent DGK z -immunoreactivity in the collecting and distal tubules among cell nuclei of most other cells in the renal parenchyma showing more or less immunoreactivity.

Regarding the nuclear localization of DGK ζ, two (KG and HK) of the present authors were the first to identify a nuclear localization sequence (NLS) in the primary molecular structure of DGK ζ (Goto & Kondo, 1996). It is natural to assume first that DGK ζ in cell nuclei is involved in the regulation of cell proliferation. A previous study by other authors (Topham *et al*., 1998) has shown that constitutive nuclear localization of DGK z in cultured cells reduces the levels of nuclear DAG and attenuates cell-cycle progression through the inactivation of protein kinase C, resulting in the accumulation of cells at the Go/G1 phase of the cell cycle. It is thus possible that distinct nuclear localization of DGK ζ leads to the strict regulation of cell proliferation there. This consideration is applicable to cells of most renal tubule cells except for the proximal tubules in the cortex and the outer stripe of the outer medulla. A lack of immunoreactivity for a given molecule does not necessarily mean its non-expression but may represent its expression level lower than the detection threshold of immunohistochemistry. It can thus be safely said that the level of DGK ζ -expression and probably of its activity is low in the proximal tubule cells, which may not necessarily make the proximal tubule cells to be free of the strict regulation of cell proliferation by DGK ζ.

However, as its more likely role, it is tempting to speculate that intranuclear DGK ζ in renal tubule epithelial cells is involved in other nuclear functions such as transcriptional regulation. There have been studies showing that DGK ζ is involved in the regulation of p53, a tumor suppressor transcription factor and that its gene knockdown results in the attenuation of p53 transcriptional activity, but in the increase of p53 protein level, which leads to increased cell vulnerability to stress and feasibility of cell apoptosis in HeLa cells (Polager & Ginsberg, 2009; Tanaka *et al*., 2013, 2021). It is thus possible to consider that DGK ζ signaling strictly regulates the occurrence of apoptosis in most renal cells except for proximal tubule cells to maintain the homeostasis and integrity of the intra-renal architecture.

Regarding the apoptosis of renal tubules, the administration of gentamicin is widely recognized as a tool to induce acute kidney injury (AKI) and it is the proximal tubules on which its injury mainly affects, resulting in the predominant occurrence of apoptotic cell death in the

proximal tubule epithelia in the cortex as already reported by others (Petejova *et al*., 2020) and also by us (Hemha *et al*., 2023). It is thus reasonable to consider that such selective non-detection levels of DGK ζ as discerned in the present study causes the selective occurrence of apoptosis by AKI in the proximal tubules.

In this regard, the dominant occurrence of DGK ζ in the collecting and distal tubules is also worth noting. This finding, together with the regulation of apoptosis by DGK ζ as described above, suggests that these two tubules have a high capacity of resistance to apoptosis through DGK ζ-signaling. In addition to its prominent occurrence in nuclei, immunoreactivity for DGK ζ was discerned weakly and diffusely throughout the cytoplasm in the collecting tubule cells, different from the distal tubule cells. It should be noted here that, in addition to an NLS, a nuclear export sequence (NES) has been identified in the molecular sequence of DGK ζ (Evangelisti *et al*., 2010). Given these two molecular sequences, perhaps this DGK isozyme exerts its roles of transcriptional regulation by shuttling between the nucleus and the cytoplasm as a delicate balancer between the actions of the NLS and the NES (Goto *et al*, 2013). It remains to be elucidated how much different the possible resistance to AKI is between the collecting tubule cells and the distal tubule cells in terms of the nucleo-cytoplasmic shuttling of DGK ζ.

It is also noteworthy that the collecting tubule cells have been shown to have an established molecular mechanism for the inhibition of apoptosis by arginine-vasopressin (AVP) (Miller *et al*., 2013). AVP plays roles via its receptor composed of two species: V1 coupled with G protein Gq and V2 coupled with Gs. The principal cells of the renal collecting tubules dominantly express the V2 of AVP receptors, which is involved in regulating water and solute transport in the tubule principal cells. In their study, the V2 signal has been shown to play inhibitory roles of apoptosis through the downstream of cAMP-mediated pathway. The effect is independent of the V1 signaling-mediated inhibition of apoptosis which was formerly revealed to occur in neurons and glomerular mesangial cells in vitro (Higashiyama *et al*., 2001; Chen *et al*., 2009). From the consideration of the possible involvement of DGK z in resistance to apoptosis, it remains to be elucidated whether or not a cross-talk could occur between the V1 signal and the transcription regulation signal of DGK ζ along their cascades.

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HEMHA, P.; CHOMPHOO, S.; POLSAN, Y.; GOTO, K.; KONDO, H. & HIPKAEO, W. Localización de diacilglicerol quinasa ζ en el riñón de ratas adultas: su expresión dominante en túbulos colectores y su carencia selectiva en túbulos proximales. *Int. J. Morphol., 41(3)*:789-797, 2023.

RESUMEN: La diacilglicerol quinasa (DGK) ejerce el equilibrio del nivel intracelular entre dos segundos mensajeros, diacilglicerol y ácido fosfatídico, por su actividad de fosforilación. La DGK ζ a menudo se localiza en los núcleos celulares, lo que sugiere su participación en la regulación de las actividades intranucleares, incluidas la mitosis y la apoptosis. El presente estudio inmunohistoquímico en riñones de rata no reveló niveles de detección de inmunorreactividad de DGK ζ en los núcleos de la mayoría de los epitelios de los túbulos proximales, en contraste a la detección en los núcleos celulares de los túbulos colectores y distales, siendo el primero más dominante. Este hallazgo sugiere que DGK ζ es un factor clave que regula la vulnerabilidad a la lesión renal aguda en varios túbulos renales: su baja expresión representa la alta vulnerabilidad de las células del túbulo proximal, y su expresión distinta hace a la resistencia de las células del túbulo colector y distal. Además, esta isoenzima estaba más o menos localizada en los núcleos de las células que forman los glomérulos, así como en los núcleos endoteliales de los capilares peritubulares y otros vasos sanguíneos intrarrenales, y en los núcleos epiteliales de las cápsulas glomerulares (cápsulas de Bowman) y los cálices renales, incluidas las células intersticiales intrarrenales.

PALABRAS CLAVE: DGK ζ ; Riñón, Rata; Núcleo; Túbulo renal.

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