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Aldosterone rapidly activates p-PKC delta and GPR30 but suppresses p-PKC epsilon protein levels in rat kidney

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Objectives. Aldosterone rapidly enhances protein kinase C (PKC) alpha and beta1 proteins in the rat kidney. The G protein-coupled receptor 30 (GPR30)-mediated PKC pathway is involved in the inhibition of the potassium channel in HEK-239 cells. GPR30 mediates rapid actions of aldosterone *in vitro*. There are no reports available regarding the aldosterone action on other PKC isoforms and GPR30 proteins *in vivo*. The aim of the present study was to examine rapid actions of aldosterone on protein levels of phosphorylated PKC (p-PKC) delta, p-PKC epsilon, and GPR30 simultaneously in the rat kidney.

Methods. Male Wistar rats were intraperitoneally injected with normal saline solution or aldosterone (150 μg/kg body weight). After 30 minutes, abundance and immunoreactivity of p-PKC delta, p-PKC epsilon, and GPR30 were determined by Western blot analysis and immunohistochemistry, respectively.

Results. Aldosterone administration significantly increased the renal protein abundance of p-PKC delta by 80% ($p<0.01$) and decreased p-PKC epsilon protein by 50% ($p<0.05$). Aldosterone injection enhanced protein immunoreactivity of p-PKC delta but suppressed p-PKC epsilon protein intensity in both kidney cortex and medulla. Protein abundance of GPR30 was elevated by aldosterone treatment ($p<0.05$), whereas the immunoreactivity was obviously changed in the kidney cortex and inner medulla. Aldosterone translocated p-PKC delta and GPR30 proteins to the brush border membrane of proximal convoluted tubules.

Conclusions. This is the first *in vivo* study simultaneously demonstrating that aldosterone administration rapidly elevates protein abundance of p-PKC delta and GPR30, while p-PKC epsilon protein is suppressed in rat kidney. The stimulation of p-PKC delta protein levels by aldosterone may be involved in the activation of GPR30.

Key words: aldosterone, rapid action, p-PKC delta, p-PKC epsilon, GPR30, protein abundance, immunohistochemistry, rat kidney

Aldosterone has been shown to have rapid nongenomic effects in a variety of tissues (Grossmann and Gekle 2009; Thomas and Harvey 2011; Dooley et al. 2012). This process acts rapidly (≤30 min) and is insensitive to transcription or translation inhibitors (Grossmann and Gekle 2009; Thomas and Harvey 2011; Dooley et al. 2012). Protein kinase C (PKC) is one of the crucial signal transductions activated by rapid actions of aldosterone (Thomas et al. 2007). It has been shown that aldosterone rapidly elevates PKC

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alpha and PKC beta1 in rat kidney (Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). However, no data are available to demonstrate the rapid effects of aldosterone on other PKC isoforms such as PKC delta or PKC epsilon. A previous *in vitro* study in cardiac myocytes has shown that aldosterone administration increases $Na^+/K^+/2Cl^-$ cotransporter activity within 15 min mediated by PKC epsilon (Mihailidou et al. 2004). In addition, G protein-coupled receptor 30 (GPR30) mediates rapid actions of aldosterone *in vitro* (Gros et al. 2011a; Brailoiu et al. 2013). The GPR30-mediated PKC pathway is involved in the inhibition of potassium channel in HEK-239 cells (Dong et al. 2013). There are no *in vivo* studies available regarding the rapid actions of aldosterone on other PKC isoforms (delta and epsilon) or GPR30 proteins.

The present study is aimed to investigate rapid alterations induced by aldosterone on the protein levels of phosphorylated PKC (p-PKC) delta, p-PKC epsilon, and GPR30 simultaneously in the rat kidney. The protein abundance of p-PKC (delta and epsilon), and GPR30 were measured by Western blotting 30 min after aldosterone injection. The localization and distribution of these proteins were examined by immunohistochemistry.

Materials and methods

Animals. Male Wistar rats 6–8 weeks old, weighing 200–240 g, and pathogenically free, were obtained from the National Center of Scientific Use of Animals, Mahidol University, Nakornpathom, Thailand. The rats were housed in a room with controlled temperature (23±1 °C), 12-hour light/dark cycle. Animals were fed with a regular rat chow (dry pellets) and tap water ad libitum (Sinphitukkul et al. 2011; Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). The rats were acclimatized for 3 days before starting the experiment. All the animal protocols were approved by the Ethics Committee of Research, Chulalongkorn University (Permit number IRB 03/56). The serum creatinine of each rat should be < 1 mg/dl (Sinphitukkul et al. 2011; Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017).

Experimental design. The rats were divided into two groups (n=6/group): vehicle (normal saline solution; NSS: 0.5 ml/kg body weight (b.w.) by intraperitoneal injection (i.p.); and Aldo (aldosterone 150 μg/kg b.w., diluted in NSS, i.p.; Sigma, St. Louis, MO, USA) (Sinphitukkul et al. 2011; Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). We used this dose as previously performed in studies on rapid actions of aldosterone on the protein levels of PKCα, α_1-Na^+,K^+-ATPase, PKCβ (I and II) and NHE (1 and 3) (Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). Therefore, in the present investigation, we further examine the effect of this dose on p-PKC (delta and epsilon) and GPR30 protein levels.

On the day of the experiment, 30 min after NSS or aldosterone injection, the rats were anesthetized with thiopental (100 mg/kg b.w., i.p.) (Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). Plasma samples obtained from the abdominal aorta were stored at –80 °C until use for the measurement of aldosterone levels by a radioimmunoassay kit (Aldo-Riact; CIS Bio International, Gif-sur-Yvette, France). Blood and urine chemistry were measured by an indirect method (Model CX3; Beckman, Krefeld, Germany). The kidneys were removed, and half of each kidney was fixed in liquid nitrogen, and then stored at –80 °C until use for the measurement of p-PKC (delta and epsilon) and GPR30 protein abundance by Western blot analysis. The other half of the renal tissue was fixed in 10% paraformaldehyde, subjected to tissue processing by an automate tissue processor (Shandon Citadel 2000, Thermo Scientific, Rockford, IL, USA), and embedded into paraffin wax for localization and distribution of these proteins by immunohistochemistry (Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017).

Western blotting. Electrophoresis and immunoblotting were performed as previously described (Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). Briefly, renal tissue samples were homogenized on ice and centrifuged at 12 000 × g (Biofuge PrimoR, Heracus, Germany) for 20 min at 4 °C to remove crude debris. The supernatant parts were collected for homogenate samples. To harvest the plasma membrane, the supernatant was further centrifuged at 17 000 × g for 20 min at 4 °C. The pellet was re-suspended in buffer (Fernandez-Llama et al. 2000; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). The total protein concentrations of both homogenate samples and plasma membranes were measured as previously described (Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). The measurement of protein abundance was performed as previously described (Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). Homogenate (120 μg protein) or plasma membrane (100 μg protein) samples were mixed sample buffer and separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), and blocked in buffer containing 5% nonfat dry milk in 0.5% Tween20. The membranes were incubated with a primary poly-

clonal antibody to p-PKC delta (Ser643/676; 1:1000; Cell Signaling Technology, Danvers, MA, USA) (Parker et al. 2016), p-PKC epsilon (Ser 729: sc-12355; 1:200; Santa Cruz Biotechnology, Dallas, CA, USA) (Fan et al. 2009), GPR30 (K-19-R: sc-48524-R; 1:500; Santa Cruz Biotechnology) (Batenburg et al. 2012), or β-actin (Santa Cruz Biotechnology), followed by the respective horseradish peroxidase-linked secondary antibody (Bio-Rad). Immunoreactive proteins were detected by chemiluminescence (SuperSignal West Pico kit; Pierce), and band intensity was measured densitometrically with molecular imager ChemiDoc XRS system (Bio-Rad). The relative protein levels of p-PKC (delta and epsilon) and GPR30 in each sample were present as a percentage of the control normalized to its β-actin content.

Immunohistochemitry. Detection of protein localization was performed as previously described (Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017). Paraffin-embedded kidney was cut into 4 μm thick sections. The sections were deparaffinized and endogenous peroxidase was blocked by treatment with 3% H_2O_2. The sections were then incubated with the primary antibody to p-PKC delta (1:1000; Cell Signaling), p-PKC epsilon (1:500; Santa Cruz Biotechnology), or GPR30 (1:500; Santa Cruz Biotechnology), at 4°C overnight, followed by the respective horseradish peroxidase-linked secondary antibody (Bio-Rad), and then reacted with 3, 3'-diaminobenzidine (DAB) solution (Sigma). As a negative control, the primary antibody was omitted, resulting in negative staining. Three blinded investigators independently examined and scored the staining intensity on a semi-quantitative five-tiered grading scale from 0 to 4 (0 = negative; 1 = trace; 2 = weak; 3 = moderate; 4 = strong) as previously described (Eiam-Ong et al. 2013; Eiam-Ong et al. 2017; Fujigaki et al. 2007).

Statistical analysis. The results of renal p-PKC (delta and epsilon) and GPR30 protein abundance were expressed as the mean ± SD. Student's t-test for independent groups was used to analyze statistical differences. A p-value of <0.05 was considered statistically significant. Statistical tests were analyzed using SPSS program version 22.0 (SPSS Inc., Chicago, IL, USA). The median staining intensity (score) of renal p-PKC (delta and epsilon) and GPR30 protein levels was presented as previously described (Eiam-Ong et al. 2013; Eiam-Ong et al. 2014; Eiam-Ong et al. 2017).

Table 1
Blood and urine chemistry in experimental groups.

Parameter	Vehicle	Aldo
Plasma aldosterone (pmol/l)	1 522.11±12.45	6 408.13±98.18*
Plasma sodium (mmol/l)	139.33±1.36	141.22±5.82
Plasma potassium (mmol/l)	3.73±0.12	3.66±0.28
Plasma chloride (mmol/l)	101.14±2.14	102.44±2.67
Plasma bicarbonate (mmol/l)	24.52±1.54	25.02±2.08
Plasma creatinine (mg/dl)	0.24±0.02	0.24±0.02
Blood urea nitrogen (mg/dl)	20.12±1.38	21.12±3.18
Ratio of plasma sodium to potassium	41.48±3.94	40.82±2.88
Ratio of urinary sodium to potassium	0.32±0.02	0.33±0.02

Data are expressed as means ± SD, n=6/group. *p<0.001 vs. vehicle.

Results

Blood and urine biochemical results. As shown in Table 1, plasma aldosterone levels significantly increased in the Aldo group compared with the vehicle group (p<0.001, df=10). There were no significant changes in plasma sodium, potassium, chloride, bicarbonate, creatinine, or blood urea nitrogen between vehicle and Aldo groups. Aldosterone administration did not significantly alter the ratio of plasma sodium to potassium or the ratio of urinary sodium to potassium compared with vehicle group.

Protein abundance of renal p-PKC (delta and epsilon) and GPR30. By Western blot analysis (Figure 1), the protein levels of p-PKC delta (78 kDa), p-PKC epsilon (72 kDa), and GPR30 (38 kDa) were assessed. Aldosterone administration enhanced protein abundance of renal p-PKC delta from 100% in the vehicle group to 180±9% (p<0.01), but p-PKC epsilon protein declined to 52±4%, p<0.05 (n=6/group, df=10). GPR30 protein abundance was elevated by aldosterone treatment from 100% in the vehicle group to 135±11%, p<0.05 (n=6/group, df=10).

Protein localization of renal p-PKC delta. The protein localization of p-PKC delta in the cortex of the vehicle group is demonstrated in Figure 2B and Table 2. The immunoreactivity was trace in the glomerulus (GL) and the proximal convoluted tubule (PCT). The staining revealed strong level in the cortical collecting duct (CCD) and moderate level in the peritubular capillary (Pcap). No staining was

Table 2
Median staining intensity (score) of renal p-PKC delta, p-PKC epsilon and GPR30 protein localization.

Region	Median staining intensity (score)					
	p-PKC delta		p-PKC epsilon		GPR30	
	Vehicle	Aldo	Vehicle	Aldo	Vehicle	Aldo
Cortex						
GL	1	1	1	1	1	1
PCT	1	3	1	0	2	3
DCT	0	0	0	0	2	2
CCD	4	4	4	1	3	2
Pcap	3	3	1	1	1	1
Outer Medulla						
TALH	1	2	1	0	0	1
MCD	2	4	1	0	1	1
VR	1	2	1	1	0	1
tLH	1	1	1	1	0	1
Inner Medulla						
MCD	2	2	2	1	1	1
VR	1	2	2	2	1	3
tLH	1	2	2	1	1	3

Staining intensity: 0 = negative, no reactivity; 1 = trace, faint or pale brown staining with less membrane reactivity; 2 = weak, light brown staining with incomplete membrane reactivity, 3 = moderate, shaded of brown staining of intermediate darkness with usually almost complete membrane reactivity; 4 = strong, dark brown to black staining with usually complete membrane pattern, producing a thick outline of the cell (Eiam-Ong et al. 2017).
Abbreviations: GL – glomerulus; PCT – proximal convoluted tubule; DCT – distal convoluted tubule; CCD – cortical collecting duct; Pcap – peritubular capillary; TALH – thick ascending limb of the loop of Henle; MCD – medullary collecting duct; VR – vasa recta; tLH – thin limb of the loop of Henle (n=5/ group).

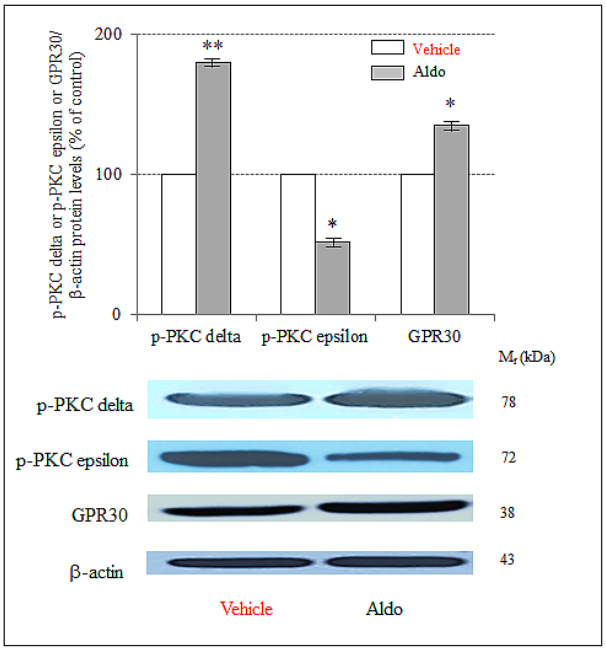

Figure 1. Western blot analysis of renal p-PKC delta, p-PKC epsilon, and GPR30 protein abundance in vehicle and Aldo groups. Histogram bars show the densitometric analyses ratios of p-PKC delta, p-PKC epsilon or GPR30 to β-actin intensity, and the representative immunoblot photographs are present. Data are means+SD of 6 independent experiments. *p<0.05, **p<0.01 vs. vehicle.

noted in the distal convoluted tubule (DCT). Aldosterone treatment increased the intensity score only in the PCT from 1 to 3 with the obvious staining at the brush border membrane (Figure 2C). Aldosterone injection did not change the intensity score in the CCD but translocated the immunoreactivity from the cytosolic compartment to the plasma membrane area.

In the outer medulla (OM), aldosterone administration elevated the intensity score from 2 to 4 in the medullary CD (MCD), whereas staining in the thick ascending limb of the loop of Henle (TALH), and vasa recta (VR) was increased to weak levels (Figure 2F and Table 2). In the inner medulla (IM), aldosterone treatment enhanced immunoreactivity to weak levels in the VR and tLH, but the intensity score in the MCD did not change. (Figure 2I). Aldosterone injection obviously induced immunostaining at plasma membranes of the MCD (Figures 2F, 2I).

Protein localization of renal p-PKC epsilon. The protein localization of p-PKC epsilon in the cortex in the vehicle group is shown in Figure 3B and Table 2. The immunoreactivity was trace in the GL, Pcap, and PCT, but no staining was noted in the DCT. Aldosterone administration decreased the staining score from 4 to 1 in the CCD and from 1 to 0 in the PCT (Figure 3C). The immunoreactivity in the GL and the Pcap did not change. In the OM, aldosterone treatment suppressed the intensity score from 1 to 0 in the TALH and MCD. The intensity score in the VR and tLH remained trace levels (Figure 3F and Table 2). In the IM, immunoreactivity was lowered to trace levels in the MCD and tLH by aldosterone injection (Figures 3I).

Protein localization of renal GPR30. As shown in Figure 4B and Table 2, in the cortex in the vehicle

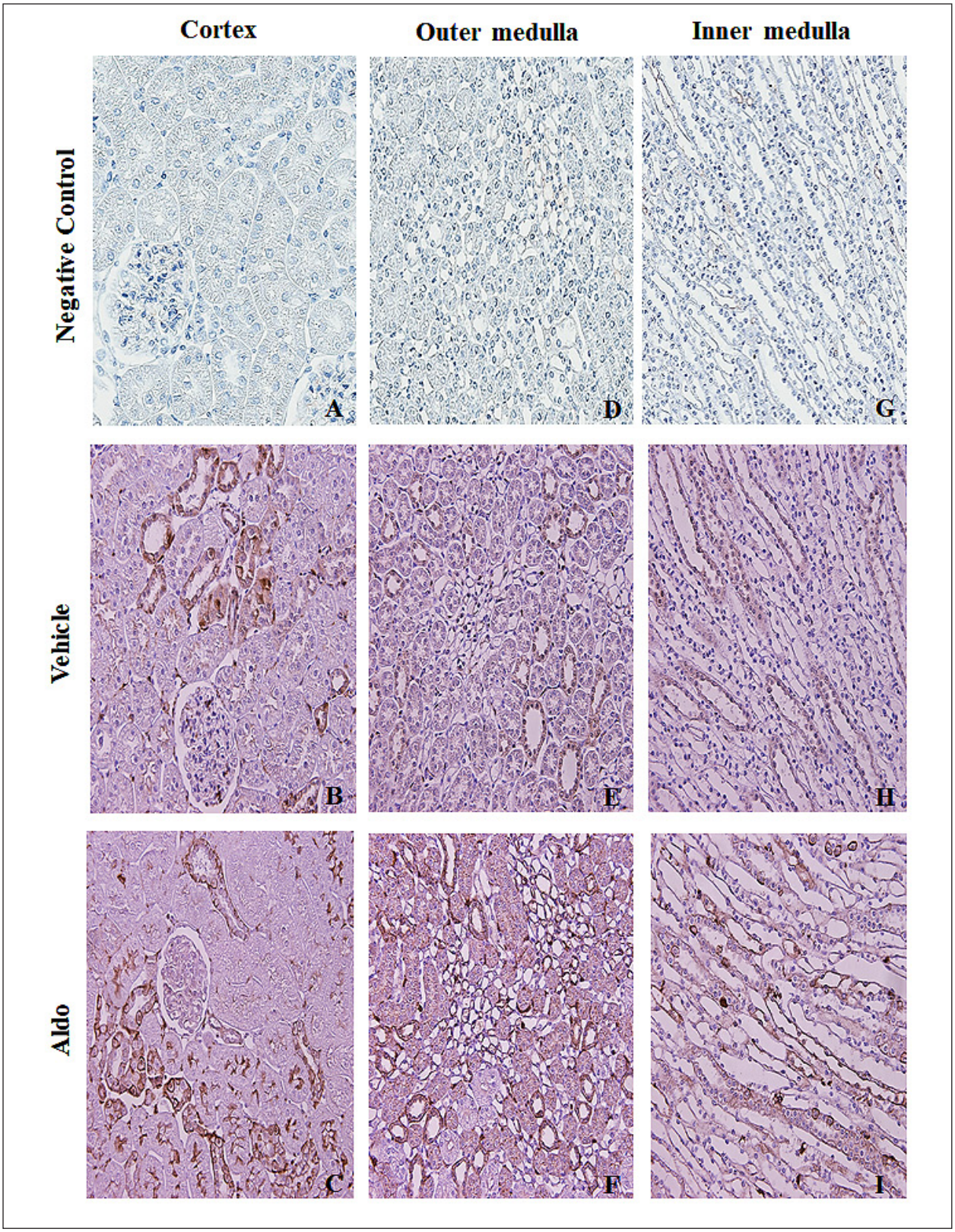

Figure 2. Representative immunohistochemical staining micrographs of renal p-PKC delta protein localization in the cortex **(A–C)**, the outer medulla **(D–F)**, and the inner medulla **(G–I)** from vehicle **(B, E, H)** and Aldo **(C, F, I)** (n=5/group). Negative controls **(A, D, G)**. Original magnification, ×400 **(A–C)** and ×200 **(D–I)**.

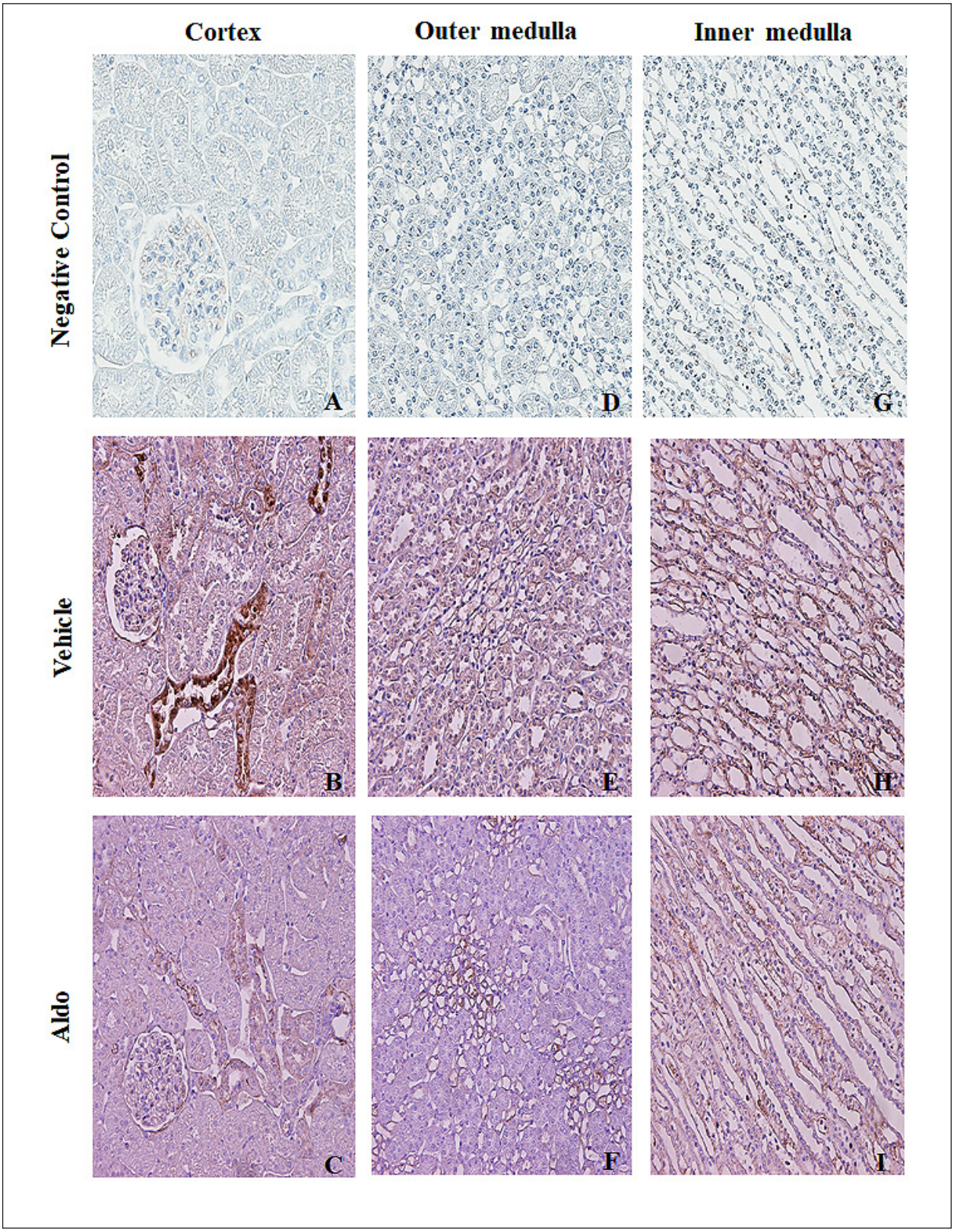

Figure 3. Representative immunohistochemical staining micrographs of renal p-PKC epsilon protein localization in the cortex **(A–C)**, the outer medulla **(D–F)**, and the inner medulla **(G–I)** from vehicle **(B, E, H)** and Aldo **(C, F, I)** (n=5/group). Negative controls **(A, D, G)**. Original magnification, ×400 **(A–C)** and ×200 **(D–I)**.

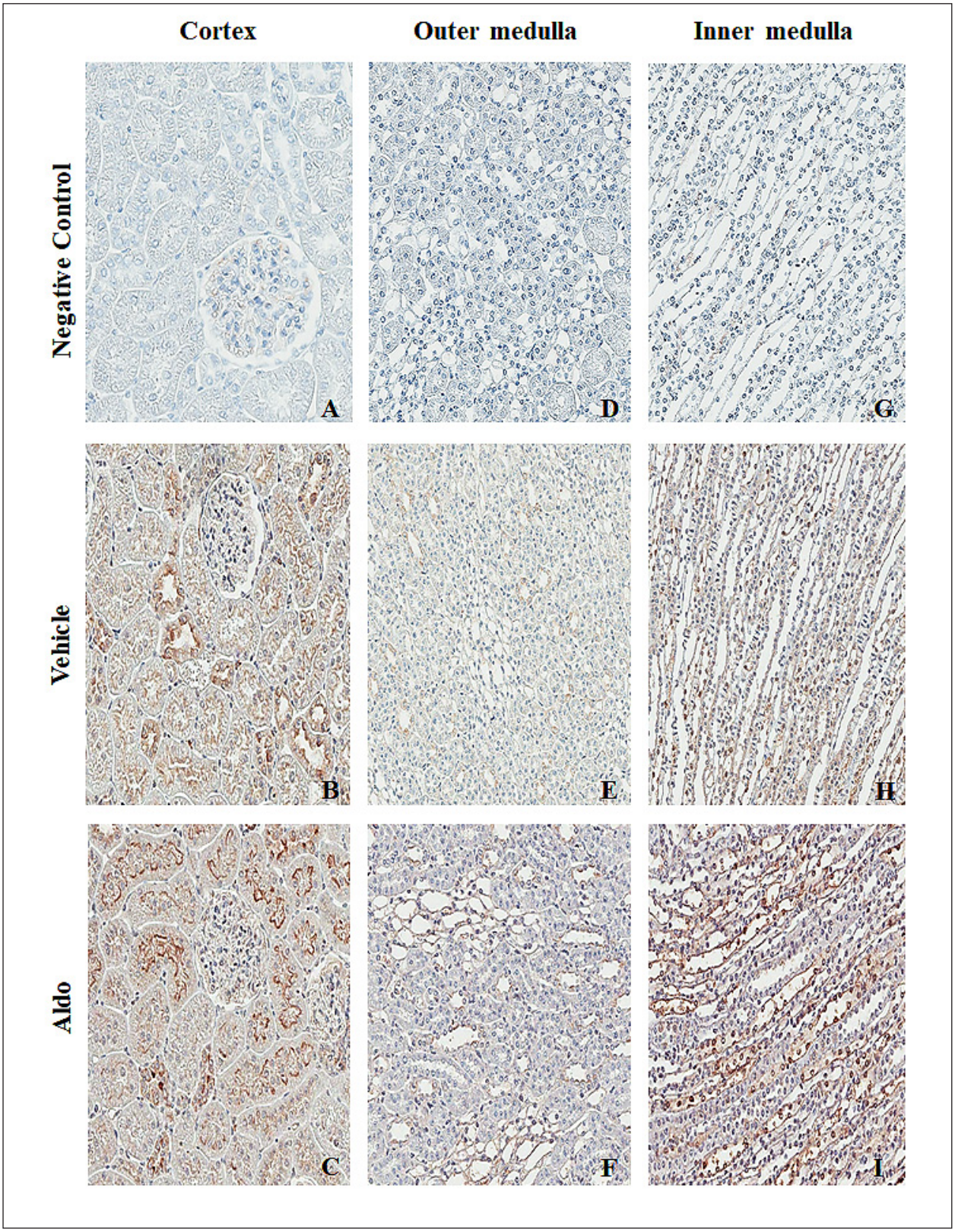

Figure 4. Representative immunohistochemical staining micrographs of renal GPR30 protein localization in the cortex **(A–C)**, the outer medulla **(D–F)**, and the inner medulla **(G–I)** from vehicle **(B, E, H)** and Aldo **(C, F, I)** (n=5/group). Negative controls **(A, D, G)**. Original magnification, ×400 **(A–C)** and ×200 **(D–I)**.

group, the immunoreactivity of renal GPR30 protein showed trace diffuse staining in the GL and the Pcap. The weak diffuse staining was observed in the PCT and DCT, whereas the staining in the CCD was moderate (Figure 4B). Aldosterone administration increased the intensity score at the luminal side of the PCT from 2 to 3, but reduced the score from 3 to 2 in the CCD (Figure 4C). The immunoreactivity in the GL, Pcap, and DCT was unchanged by aldosterone treatment. In the OM, aldosterone injection increased the staining to trace levels (score=1) in the TALH, VR, and tLH, but the staining was unchanged in the MCD (Figure 4F). In the IM, immunoreactivity was elevated to moderate levels in the VR and the tLH by aldosterone treatment while the intensity score in the MCD was unchanged (Figure 4I and Table 2).

Discussion

The present results provide the first *in vivo* data simultaneously showing renal p-PKC delta, p-PKC epsilon, and GPR30 protein abundance as well as immunoreactivity 30 minutes following aldosterone administration. Aldosterone treatment significantly enhanced the renal protein abundance of p-PKC delta by 80%, whereas p-PKC epsilon protein tended to reduce (Figure 1). Activation of PKC family is a notable consequence of rapid effects induced by aldosterone (Thomas et al. 2007). This may explain by aldosterone-induced transient increases in intracellular Ca^{2+} levels, which then activate Ca^{2+}-dependent PKCs (Harvey and Higgins 2000; Le Moellic et al. 2004). We previously demonstrated in the rat kidney that aldosterone injection for 30 minutes increased protein abundance of PKCα and enhanced immunoreactivity staining of PKCα in the PCT with the translocation from basolateral to luminal membranes (Eiam-Ong et al. 2014). Recently, we have shown that aldosterone injection for 30 min also elevated protein abundance and immunostaining intensity of PKCβI in the rat kidney (Eiam-Ong et al. 2017).

Protein kinase C (PKC) is a family of closely related serine/ threonine kinases and plays a crucial role in cellular signal transduction and cellular effector functions (Newton 1995; Schnaper 2000). PKC delta and PKC epsilon are isoforms in the new PKC (nPKC) family (Nishizuka 1988; Nishizuka 1992). There are no available data showing the rapid effect of aldosterone on these protein abundances. Our present data provide the first evidence in the rat kidney that aldosterone treatment rapidly activates p-PKC delta protein abundance but suppresses p-PKC epsilon protein level. This result implies that by contributing its rapid effect aldosterone shows diverse effects on p-PKC delta and p-PKC epsilon. These two isozymes have different and sometimes opposite effects (Duquesnes et al. 2011). PKC delta promotes apoptosis while PKC epsilon stimulates cell growth and differentiation (Duquesnes et al. 2011). The precise mechanism, by which aldosterone administration may enhance PKC delta protein abundance has not been established. PKC delta protein levels have been demonstrated to be enhanced after incubation with reactive oxygen species (Lee et al. 2005). Interestingly, aldosterone rapidly induced oxidative stress production (Queisser et al. 2011; Zhu et al. 2011). We propose that the increased p-PKC delta protein abundance in the present study may be mediated via oxidative stress induced by aldosterone. PKC delta has been shown to regulate cotransporter activity during alpha-adrenergic stimulation (Liedtke and Cole 1997; Liedtke et al. 2001), $Na^+/K^+/2Cl^-$ activity (Liedtke and Cole 2002; Liedtke et al. 2002), and Na^+,K^+-ATPase exocytosis (Ridge et al. 2002).

For PKC epsilon, the present results show that aldosterone administration decreased p-PKC epsilon protein abundance in the rat kidney. The precise mechanism, by which aldosterone suppressed PKC epsilon protein abundance has not been clarified yet. It has been shown that in rabbit cardiomyocytes, aldosterone rapid nongenomically activates $Na^+/K^+/2Cl^-$ cotransporter activity mediated by PKC epsilon (Mihailidou et al. 2004). We speculate that it may be the tissue-specific effect of aldosterone on the suppression p-PKC epsilon the kidney. In addition, in alveolar epithelial type II cells, dopamine increased Na^+,K^+-ATPase activity through the exocytosis of the pumps from late endosomes into the basolateral membrane in a mechanism-dependent activation of PKC epsilon (Ridge et al. 2002). Moreover, PKC epsilon also contributes to regulation of the sarcolemmal Na^+,K^+-ATPase pump (Buhagiar et al. 2001).

For the immunolocalization of PKC delta and PKC epsilon, the baseline distribution of both isoforms is consistent with the previous studies (Kang et al. 1999; Redling et al. 2004). The present data show that aldosterone administration increases the immunoreactivity of p-PKC delta protein in the PCT, tLH, VR, and CD. By contrast, p-PKC epsilon immunoreactivity was suppressed in both kidney cortex and medulla areas. The present results indicate that in a rapid pathway aldosterone diversely regulates protein distribution of p-PKC delta and p-PKC epsilon along the nephron. These proteins can consequently modulate myriad renal tubular ion transport (Newton 1995; Schnaper 2014).

For GPR30, the present study shows the first time that aldosterone administration for 30 minutes increases protein abundance by 35% ($p<0.05$) (Figure 1). GPR30 belongs to the seven-transmembrane receptor (7TMR) superfamily (Filardo et al. 2006). The first receptor characterized as an "orphan G protein-coupled receptor," is expressed on the cell surface and mediates its effects via several members of the G protein family of GTP-binding proteins (Carmeci et al. 1997). The precise mechanism, by which aldosterone administration enhances GPR30 protein abundance has not been established. Despite the fact that the receptors mediating faster effects of aldosterone remained unclear, recent evidence has indicated that GPR30 may mediate the nongenomic effects of aldosterone (Feldman and Gros 2011; Funder 2011; Gros et al. 2011a; Gros et al. 2011b; Gros et al. 2013). It has been reported that aldosterone increases cardiac vagal tone via GPR30 activation (Brailoiu et al. 2013).

For GPR30 localization, the immunostaining of GPR30 protein in the vehicle group revealed a similar baseline regional distribution as previously described (Cheng et al. 2011; Lindsey et al. 2011; Cheng et al. 2014). The present data show that aldosterone administration rapidly induces GPR30 protein immunoreactivity in both the kidney cortex and medulla regions with a profound translocation of GPR30 protein to the apical membrane of proximal tubules (Figure 4C). This result implies that rapid effects of aldosterone play an important role in the regulation of renal tubular function. For example, aldosterone sensitized connecting tubule glomerular feedback via the receptor GPR30 (Ren et al. 2014). Moreover, mechanisms of connecting tubule glomerular feedback enhancement by aldosterone mediated via PKC (Ren et al. 2016). The GPR30-mediated PKC pathway is involved in the inhibition of potassium channel in HEK-239 cells (Dong et al. 2013).

Conclusion

This is the first *in vivo* study simultaneously demonstrating that aldosterone administration rapidly elevates protein levels of p-PKC delta and GPR30, but p-PKC epsilon was suppressed in the rat kidney. The stimulation of p-PKC delta protein levels by aldosterone, *per se*, may be involved in GPR30 activation.

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