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Protein Kinase D1 Modulates Aldosterone-Induced ENaC Activity in a Renal Cortical Collecting Duct Cell Line

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ABSTRACT

Aldosterone treatment of M1-CCD cells stimulated an increase in epithelial Na^+ channel (ENaC) α-subunit expression that was mainly localized to the apical membrane. PKD1 suppressed cells constitutively expressed ENaCα at low abundance, with no increase after aldosterone treatment. Here ENaCα was mainly localized proximal to the basolateral surface of the epithelium both before and after aldosterone treatment. Apical membrane insertion of ENaCβ in response to aldosterone treatment was also sensitive to PKD1 suppression as was the aldosterone-induced rise in the amiloride-sensitive, *trans*-epithelial current (I_{TE}). The interaction of the mineralocorticoid receptor (MR) with specific elements in the promoters of aldosterone responsive genes is stabilized by ligand interaction and phosphorylation. PKD1 suppression inhibited aldosterone-induced SGK-1 expression. The nuclear localization of MR was also blocked by PKD1 suppression and MEK antagonism implicating both these kinases in MR nuclear stabilization. PKD1 thus modulates aldosterone-induced ENaC activity through the modulation of sub-cellular trafficking and the stabilization of MR nuclear localization.
Key words: Aldosterone, ENaC, Protein kinase D, Renal, Cortical collecting duct, Hypertension.

INTRODUCTION

The steroid hormone aldosterone is a key regulator of electrolyte transport and blood pressure that acts upon multiple tissues and organ systems in the in the body, where its specific mineralocorticoid receptor (MR) is expressed; such target sites include the distal nephron of the kidney, the distal colon, the heart and the vascular endothelium. The principal effect of aldosterone action in renal and colonic epithelia is in the regulation of membrane transport molecules, such as the epithelial sodium channel (ENaC). ENaC activity plays a crucial role in the maintenance of whole body Na^+ homeostasis (1, 2); the abundance of ENaC along the aldosterone-sensitive distal nephron (ASDN), coupled with channel trafficking into the apical membrane of the epithelial cells facilitates and determines the rate of Na^+ re-absorption from the renal ultra-filtrate. The release of aldosterone as the last phase of the renin/ angiotensin cascade maintains tight hormonal control over ENaC expression and membrane insertion. Fully active ENaC is a heterotrimer composed of one each of the α-, β- and γ-subunits (3). Aldosterone regulates ENaC activity through multiple, synergistic mechanisms. For example, MR in complex with aldosterone acts as a liganddependent transcription factor that stimulates the tissue–specific transcriptional upregulation of ENaC subunits in the distal nephron (and in the lung); it is the ENaCα subunit that is under the direct transcriptional control of ligand-bound MR, while ENaCβ and ENaCγ are constitutively expressed or else only weakly induced by aldosterone (4). ENaC activity is also subject to indirect genomic regulation by aldosterone through increasing the stability and half-life of pre-expressed ENaC

subunits. Aldosterone suppresses the proteasomic degradation of ENaC through the serum glucocorticoid stimulated kinase (SGK-1)-dependent suppression of the ubiquitination of ENaC subunits by the E3 ubiquitin-protein ligase, Nedd4-2 (5, 6). Aldosterone also up-regulates the expression of a ubiquitin-specific protease USP2-45 to de-ubiquitinate and further stabilize pre-expressed ENaC subunits (7).

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The most rapid reported increase in amiloride-sensitive, ENaC current was detected in isolated principal cortical collecting duct (CCD) cells 2 min after aldosterone treatment (8); the signalling cascade responsible for increased ENaC activity in this model remains unknown. Other experimental systems may provide a clue as to the mechanism behind this rapid increase in ENaC activity; such as the elevation of apical membrane ENaC density that is coupled to the stimulation of RhoA-mediated vesicle trafficking (9). The modulation of the rates of membrane insertion and subcellular trafficking may thus be important additional mechanisms for ENaC regulation by aldosterone (10). While the increase in ENaC activity following aldosterone treatment is more typically detected several hours after hormone treatment and is associated with the direct and indirect transcriptional effects through MR; rapidly activated signalling cascades may serve to potentiate or amplify these effects. The rapid activation of signalling cascades by aldosterone in cells of renal origin has been described by numerous groups [reviewed in (11)], and these rapid responses have variously been described as MR-dependent or MR-independent [reviewed in (12)]. The role of aldosterone-induced rapid signalling responses in modulating the key physiological responses to aldosterone such as renal Na^+ re-absorption or K^+ secretion is still unclear.

We recently demonstrated that aldosterone stimulated the activation of protein kinase D (PKD)1 in the M1-CCD cell line through an EGFR-coupled signalling cascade that was initiated by the interaction of aldosterone with MR (13). PKD1 is the prototypic member of the PKD family of serine/ threonine protein kinases, and is activated in response to diverse extra-cellular stimuli (14) to regulate crucial cellular processes including cell growth and apoptosis [reviewed in (15)]. Each of the PKD family isoforms have also been implicated in different aspects of the regulation of subcellular trafficking, either through the maintenance of Golgi structure or by regulating

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TGN fission and Golgi to membrane vesicle trafficking through the activation of PI4KIII β (16-18). The RhoA-mediated translocation of ENaC to the cell membrane is coupled to vesicle trafficking through the activation of PI4P5K (9). Interestingly, the ENaC subunits are also known to interact directly with phosphatidylinositide signalling intermediates (19), and PKD1 itself can be activated as a consequence of stimulating RhoA-coupled signalling cascades (20). We have shown that PKD1 activation by aldosterone in M1-CCD cells played a role in early, sub-cellular trafficking events that resulted in the redistribution of eCFP-tagged ENaC subunits within a few minutes of aldosterone treatment (21). These findings are advanced in this present study through the investigation the role of aldosterone-induced PKD1 activation in regulating aldosterone-induced ENaC activity, and the sub-cellular distribution of ENaC subunits in M1-CCD cells. The time frame analysed in this present study is coincident with detectable increases in ENaC-dependent transepithelial electrical current (I_{TE}), and we found that suppression of PKD1 expression inhibited both the apical translocation of ENaC subunits and the increase in amiloride-sensitive I_{TE} stimulated by aldosterone treatment.

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MATERIALS AND METHODS

Cell culture and reagents

The M1-CCD cell line (ATCC, CRL-2038) was derived from renal CCD microdissected from a mouse transgenic for the early region of SV40 virus (strain

Tg(SV40E) Bri7) (22). M1-CCD cells were routinely grown in 75 cm^2 polystyrene culture flasks containing 1:1 Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM:F-12) with fetal bovine serum (10%); supplemented with L-glutamine (2 mM), penicillin (100 $units.ml^{-1}$), streptomycin (100 $\mu g.ml^{-1}$) and dexamethasone (5 μM). Cultures were maintained in an atmosphere of 70% humidity, 5% CO_2 at 37^oC. Culture medium was changed every 2 to 3 days, and cells were sub-cultured by trypsinization before they became confluent. For experimental purposes, M1-CCD cells were propagated on 13 mm diameter Millicell-HA semi-permeable supports (Millipore), in 8-well glass chamber slides (Nunc) or 10 cm dia. culture dishes as required; experiments were performed once a fully confluent monolayer had been established. Aldosterone (Steraloids) was prepared as a 50 mM stock in ethanol; then further diluted in culture medium, so that the final concentration was 10 nM. Vehicle controls were the equivalent concentration of ethanol diluted in culture medium. All other chemical reagents used in this investigation were purchased from Sigma-Aldrich, unless otherwise indicated.

Development of PKD1 siRNA knock-down cell line

An M1-CCD cell line was developed that stably expressed a PKD1-specific siRNA as previously described (21). Briefly, oligonucleotides were designed to facilitate the synthesis of DNA expression cassettes that expressed PKD1-specific siRNA molecules, under the control of the murine U6 promoter using the Silencer Express system (Ambion). The efficacy of each of the expression cassettes in suppressing PKD1 expression was verified by Western blotting using a PKD1 specific antibody.

Expression cassettes that effectively suppressed PKD1 expression were sub-cloned between the *Eco*R1 and *Hin*d *III* sites of the pSEC-neo mammalian expression vector (Ambion). Recombinant plasmids were transfected into M1-CCD cells using Lipofectamine 2000 (Invitrogen). Neomycin resistant clones harbouring the plasmid developed into colonies following propagation in the presence of G418, and were isolated (21). Cell numbers were amplified in selective medium, and all experiments using the PKD1 stable knock down cell line were performed in the presence of G418. Continued suppression of PKD1 expression in the knockdown cell line was verified by Western blotting using the PKD1-specific antibody.

Confocal immunofluorescence imaging

M1-CCD cells were grown on 13 mm cover slips until a confluent monolayer was established. After treatment with aldosterone as indicated; the cell monolayer was washed in ice-cold PBS and fixed in 4% paraformaldehyde in PBS. The cell membranes were disrupted in 0.2% Triton X-100 in TBS, and the non-specific binding of antibodies and fluorescent conjugates were blocked by incubation in 2% gelatin as above. Specific rabbit polyclonal antibodies were used to detect ENaCα (Chemicon) and ENaCβ (Abcam); MR was detected using a specific murine mAb, MRN 4E4 (23). Bound primary antibodies were detected using a goat anti-mouse Alexa488 conjugate (Invitrogen), or a goat anti-rabbit Alexa488 conjugate (Invitrogen) as appropriate. Cells were counter stained with TRITC labelled phalloidin for the detection of polymerized actin, or with Alexa 546 labelled wheat germ agglutinin (WGA) for detection of the cell membrane as indicated. Labelling of the cell membrane with Alexa546-WGA was performed in advance of cell membrane disruption, in order to stain the apical membrane only. Cells were mounted in Vectashield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI), and examined using a LSM 510 Meta confocal microscope. Laser excitation

wavelengths for DAPI Alexa Fluor 488 Alexa 546 and TRITC were 361 nm, 488 nm 543 nm and 543 nm respectively. Images were captured at x 40 magnification and x4 zoom. Scans were performed at 1μm interval depths through the fixed cells, and single or merged images are presented as XY single planes through the mid-section

of the cells unless otherwise specified. Determination of ENaCβ membrane insertion was captured at x63 magnification; XZ planes show apical to basolateral crosssections of the labelled cell monolayers.

Trans-epithelial current measurements

Wild-type M1-CCD cells and M1-CCD cells suppressed in PKD1 expression were seeded at a density of 1 x10_5 cells per well onto 0.6 cm semi-permeable supports, and maintained in DMEM:F12 medium supplemented as above. The medium was changed every 48 h after seeding and the trans-epithelial resistance (R_{TE}) of the developing monolayer was measured daily, using an EVOM (World Precision Instruments) Ohmeter connected to STX electrodes. The R_{TE} of wild-type and PKD1 suppressed M1-CCD cells were compared over 7 days. The development of a high resistance epithelium, as determined by changes in R_{TE} by the M1-CCD cells, was not significantly affected by PKD1 suppression. Once high resistance had been established (>1 kΩ), the culture medium was substituted with serum free medium containing 1 μM dexamethasone for 6 h, this did not significantly affect R_{TE}. The epithelial monolayers were subjected to antagonist and hormone treatments for the times indicated. R_{TE}; trans-epithelial potential difference (V_{TE}) measurements were recorded, and I_{TE} was calculated using Ohm's law ($V_{TE}/I_{TE} = R_{TE}$). The experiment was repeated 3 times and the data shown are from one representative experiment, expressed as mean $I_{TE} \pm$ S.E.M. measurements of n= 8 separate cell monolayers at each time point with I_{TE} values expressed as μA/cm_2.

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Preparation of cell lysates and sub-cellular fractions

For experimental purposes, wild-type M1-CCD and PKD1 suppressed M1-CCD cells were seeded into 10 cm dishes, grown to 80% confluency and a quiescent state was induced through growth in serum free medium for 24 h. Cells were incubated with aldosterone (10 nM) or vehicle control for periods indicated. Following treatment, the cell monolayers were washed three times with ice cold PBS and collected in 1 ml PBS. To obtain cell lysates, cell suspensions were centrifuged at 4_oC for 30 s at 2000 *xg* and the supernatant was removed. The cell pellet was lysed with the addition of lysis buffer (20 mM Tris-HCl pH 7.6, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 1 mM of dithiothreitol, 10 μg.ml_{-1} aprotinin, 10 μg.ml_{-1} leupeptin, 10 mM NaF, 1 mM phenylmethylsufonylfluoride and 0.5% v/v NP40). Insoluble material was isolated by centrifugation at 4_oC for 15 min at 20 000 *xg*. For nuclear/ cytoplasmic sub-cellular fractionation, isolated cells were re-suspended in extraction buffer A (1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 10mM HEPES, pH 7.9) and centrifuged at 20 000 *xg* for 10 min. The pellets were resuspended in a further 50 μl buffer A that was supplemented with IGEPAL (0.1%), centrifuged as above and the supernatant containing the cytoplasmic proteins was isolated. The insoluble material was combined with 50 μl extraction buffer B (420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % Glicerol, 20 mM HEPES pH 7.9) and re-suspended by vigorous vortexing before centrifugation as above. The supernatant from this second lysis step comprised the nuclear fraction. The protein concentration of each of the lysates was determined and standardized by Bradford Assay (Bio-Rad).

SDS-PAGE and Western blotting

Cell lysates were dissolved by boiling in Laemmli sample buffer and proteins were separated by SDS-PAGE on 10% w/v polyacrylamide gels. The proteins were transferred onto PVDF membranes, and probed with the specified primary antibodies

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diluted in 5% w/v bovine serum albumin (BSA) in TBS according to the supplier's instructions. Bound antibodies were detected using anti-mouse or anti-rabbit horseradish peroxidase secondary conjugates; antibody-labeled proteins were visualized by enhanced chemiluminescence, ECL (Amersham Biosciences). Exposed films were digitally photographed and subjected to densitometric analysis

using GeneSnap software (Synoptics). ENaC sub unit antibodies and the MR-specific antibody were as above. The SGK-1 antibody and laminin antibody were obtained from Santa Cruz Biotechnology and Cell Signalling respectively. The β-actin antibody, anti-mouse and anti rabbit IgG horseradish peroxidase secondary conjugates were obtained from Sigma-Aldrich.

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RESULTS

Aldosterone-induced ENaCα subunit expression in M1-CCD cells is PKD1-dependent

ENaC activity is subject to regulation by aldosterone through different mechanisms including the transcriptional regulation of subunit expression, and the modulation of subunit ubiquitination; which affects ENaC abundance at sub-apical, cytoplasmic pools. These inactive pools of ENaC can be recruited to the apical membrane in response to specific agonists and the activation of their associated secondary messenger systems (9). We have previously shown that aldosterone treatment stimulates the rapid sub-cellular redistribution of pre-expressed, eCFP-tagged ENaC subunits (21). In this present study, the effect of aldosterone treatment over more extended periods on the sub-cellular localization and abundance of ENaCα was investigated by immunofluorescence in order to lend greater physiological relevance to our original findings. This experimental approach also allowed a differentiated, confluent epithelium of M1-CCD cells to be used as an experimental model, and avoided the physiological consequences of constitutive ENaC subunit overexpression when using heterologous expression plasmids. In untreated M1-CCD cells, ENaCα was present in the cytoplasm at barely detectable levels (Fig. 1A); while after 24 h aldosterone treatment the ENaCα subunit increased in expression relative to the untreated control. Here ENaC was detected as punctuate immunofluorescent staining of the cytoplasm, suggesting the localization of ENaC to distinct cytoplasmic vesicles. ENaC was also abundant in the confocal XY plane above the perijunctional filamentous actin ring, suggesting that a sub-set of the ENaCα was localized to sites proximal to the apical membrane. Conversely, there was no ENaCα immuno-staining in the most basolateral XY confocal plane, suggesting a lack of ENaCα in this region of the epithelial cells.

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We have previously demonstrated that PKD1 is significantly activated (3-fold above basal levels) in aldosterone treated M1-CCD cells (13), and that PKD1 activation is also involved in the rapid translocation of ENaC subunits observed within 2 min of aldosterone treatment in the M1-CCD cells (21). The elucidation of the role of PKD1 in these aldosterone responses was facilitated by deriving a M1-CCD cell line clone that stably expressed a PKD1-specific siRNA, and which achieved 80-90% suppression of PKD1 protein expression. In this cell line ENaCα was expressed at a higher basal level of abundance than was observed in the wild-type cells (Fig. 1). In addition, after 24 h aldosterone treatment, ENaCα abundance was not altered in the PKD1 suppressed cells and ENaCα remained localized to densely staining sites in the cytoplasm, which were basolateral relative to the perijunctional filamentous actin ring. The increase in ENaCα abundance after aldosterone treatment was thus dependent on PKD1 expression and activation, as was the association of ENaC with the apical membrane (Fig. 1B). These data suggest a dual role for PKD1 in the regulation of ENaC activity by aldosterone through the modulation of MR-dependent gene expression, and also through the modulation of sub-cellular trafficking.

Membrane insertion of ENaC is PKD1-dependent

The increase in ENaC abundance in the cell membrane following aldosterone treatment is the product of a combination of processes that influence ENaC subunit expression, stability and trafficking. Recent investigations have implicated each of the PKD isoforms in the regulation of vesicle fission from the TGN to facilitate Golgi to

cell membrane trafficking (24). Consequently, the insertion of ENaC into the cell membrane following aldosterone treatment may be influenced by the rate of post-Golgi vesicle fission that is regulated by the PKD family proteins. In order to distinguish the effects of PKD1 activation on ENaC subunit trafficking as opposed to the gene expression effects, ENaCβ membrane insertion was investigated by

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immuno-fluorescence before and after 24 h aldosterone treatment. The ENaCβ subunit is not subject to transcriptional control by aldosterone in the distal nephron in the manner of ENaCα, and allows some discrimination to be made between the dual effects of PKD1 activation. The apical membrane of the cells making up the intact, confluent M1-CCD cell monolayer was labelled using Alexa546 conjugated to WGA; the tight junction complexes of live, confluent, epithelial cells prevent access of the complex to sub-apical membranes when staining is performed prior to fixation, resulting in staining of the apical membrane only. We observed that in wild-type cells ENaCβ was located in the cytoplasm with greater basolateral localization, and no colocalization

with the WGA, suggesting low apical membrane abundance (Fig. 2).
However, following aldosterone treatment for 24 h, there was a diminished basolateral localization of ENaCβ and increased insertion of ENaCβ into the apical membrane as indicated by WGA co-localization. In the PKD1 suppressed cells, ENaCβ was localized to the cytoplasm and there was no increase in apical membrane abundance following aldosterone treatment for 24 h. The pattern of localization of ENaCβ in the cytoplasm without aldosterone treatment also differed between the wild-type and knock-down cells. ENaCβ expressed by wild-type cells was localized to densely staining, punctate structures within the cytoplasm; while the ENaCβ expressed by PKD1 suppressed cells was evenly distributed through the cytoplasm of the cells. These data demonstrate that PKD1 expression influenced the distribution of ENaCβ to vesicular structures in advance of aldosterone treatment, and also determines the apical membrane insertion of ENaC subunits following aldosterone treatment.

Aldosterone stimulation of the amiloride-sensitive I_{TE} is PKD1-dependent

PKD1 activation is implicated in post-Golgi protein trafficking, and the establishment of a high R_{TE} by epithelial cells is dependent upon the secretion of occlusion junction

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proteins into the intercellular space. Suppression of PKD1 expression may be anticipated to have a deleterious effect on the establishment of high R_{TE} by a monolayer of M1-CCD cells through the perturbation of protein trafficking. To investigate this possibility, M1-CCD cells and cells suppressed in PKD1 expression were seeded onto semi-permeable supports, and the R_{TE} was measured and recorded daily over the course of 7 days (Fig. 3A). The data is presented ± SEM and was obtained by measuring R_{TE} of four individual monolayers for each cell line, and is representative of three independent experiments carried out on different days. Wildtype cells developed maximum electrical resistance (1.38 ± 0.12 kΩ) 7 d after seeding; when seeded at an identical density (1 x 10^5 per well) the PKD1 suppressed cells developed maximum electrical resistance (1.63 ± 0.08 kΩ) 6 d after seeding. The R_{TE} of wild-type and PKD suppressed cells was not significantly different, and the R_{TE} remained stable for 3 to 4 d after the R_{TE} maxima were reached.
M1-CCD cells develop high R_{TE} that is equivalent to that measured for the primary CCD making them a suitable model for investigating the physiology of the CCD, and in particular the effects of aldosterone (25). Aldosterone stimulates an amiloridesensitive increase in ENaC current in M1-CCD cells 2 to 4 h after treatment (25) and we found that in the presence of 1 μM dexamethasone, wild-type M1-CCD cells and M1-CCD cells suppressed in PKD1 expression had a basal I_{TE} of 5 μA/cm^2 (Fig. 3B). However, following aldosterone treatment, the wild-type cells generated a significant

rise in I_{TE}, increasing to 6 μA/cm^2 after 2 h, which further increased to 10 μA/cm^2 4 h after aldosterone treatment. This increase in I_{TE} was stable and sustained for at least 24 h after hormone treatment, when the I_{TE} was measured at 11 μA/cm^2 and amiloride treatment caused the I_{TE} to rapidly drop back to 4 μA/cm^2. Thus basal ENaC activity contributed a current of 1 μA/cm^2 to the total I_{TE} that increased to a 7 μA/cm^2 contribution 24 h after aldosterone treatment. In comparison aldosterone
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stimulated a small increase in I_{TE} in the PKD1 suppressed cells, which was maximal after 4 h at 7 μA/cm^2 as compared to 10 μA/cm^2 for the wild-type cells over the 4 to 24 h time period. This increase in I_{TE} at 4h by the PKD1-suppressed cells was not sustained, and the amiloride-sensitive component of the stimulated I_{TE} was 2 μA/cm^2 as compared to 7 μA/cm^2 for the wild-type cells. These data demonstrate that aldosterone stimulates a 7-fold increase in the amiloride-sensitive, ENaC component of the total I_{TE} in the M1-CCD cells that is greatly attenuated when the expression of PKD1 is suppressed.

The stimulation of an ENaC current in the M1-CCD cells was only detectable when the cells were treated with aldosterone in combination with dexamethasone. Aldosterone treatment alone did not stimulate a detectable change in I_{TE} and it has been noted by other workers that M1-CCD cells grown in the absence of dexamethasone fail to develop a high R_{TE} when propagated on semi-permeable supports. The measured elevation of ENaC activity in the M1-CCD cells therefore, requires concurrent MR and glucocorticoid receptor (GR) stimulation. Aldosterone alone can activate GR, but this is proposed to occur only at super-physiological concentrations of the hormone. The requirement for dexamethasone co-treatment in order to achieve the establishment of a high R_{TE} and a detectable ENaC current following aldosterone treatment in this study confirms that the aldosterone-induced effects on ENaC are mediated by MR and not by GR. However, the effects of aldosterone on ENaC activity can only be detected on a glucocorticoid-replete background.

Aldosterone-induced MR translocation is PKD1-dependent

The stimulation of ENaC activity by aldosterone over extended periods of hormone treatment is the product of the transcriptional up-regulation of ENaC subunits,
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particularly ENaCα in the distal nephron, and also the up-regulation of proteins that regulate ENaC abundance in the cell membrane such as SGK-1. Aldosterone stimulates the translocation of pre-expressed ENaC subunits (21); while full channel activity requires expression of all three subunits, and ENaCα expression in unstimulated M1-CCD cells is extremely low. Any effect of aldosterone on ENaC membrane abundance through the modulation of sub-cellular trafficking requires that the expression of ENaC subunits first to be induced. Consequently, there is a lag between hormone treatment and a change in membrane abundance, with a resulting increase in aldosterone sensitive I_{TE}. In addition to its role in modulating sub-cellular vesicle movement, PKD1 also regulates transcriptional response through its interaction with IκB kinase (IKK) (26) and ERK1/2 mitogen activated protein (MAP) kinase (27). It is well established that the modulation of gene expression by aldosterone is largely through the stabilization of the interaction of MR with target DNA sequences to promote transcription.

The change in the sub-cellular distribution of MR in M1-CCD cells treated with aldosterone was investigated using immuno-fluorescent detection with a MR-specific antibody (Fig. 4). In this present study we found that aldosterone treatment not only enhanced the association of MR with the nucleus, but also promoted the translocation of MR to discrete cytoplasmic sites proximal to the nucleus within 2 min of treatment. The increased localization of oestrogen receptor with the cell nucleus following oestrogen treatment is dependent on phosphorylation of the receptor by

ERK1/2 MAP kinase (28); while the involvement of ERK1/2 in enhancing the association of MR with the nucleus following aldosterone treatment has not previously been described. PKD1 stabilizes the activation of ERK1/2 in response to G-protein coupled receptor agonists (27), and the modulation of ERK1/2 activity may be one mechanism by which PKD1 can affect the redistribution of MR following
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aldosterone treatment. We found that this increase in MR abundance at the nucleus following aldosterone treatment, was sensitive to antagonism of the MEK/ERK cascade with PD98059 and was also inhibited in PKD1 suppressed M1-CCD cells. The synergistic actions of ERK1/2 and PKD1 thus converge to facilitate the subcellular redistribution of MR that precedes aldosterone-induced changes in gene expression.

After 30 min treatment with aldosterone, a time frame when the transcriptional effects of MR first become detectable; MR in the wild-type cells was largely localized to the perinuclear region of the cytoplasm, with some punctate staining of the nucleus (Fig. 5). In PKD1 suppressed cells, the MR was localized mainly in the cell cytoplasm, with some nuclear and perinuclear staining evident. This distribution was maintained after 60 min aldosterone treatment; however, after 120 min treatment in wild-type M1-CCD cells, MR was localized to one or two discrete accumulations adjacent to the nucleus with granular staining of the nucleus itself. In the PKD1 suppressed cells, MR was uniformly distributed throughout the cell cytoplasm with very little nuclear staining and no punctate perinuclear staining. Antagonism of the MEK/ERK cascade with PD98059 completely inhibited the aldosterone-induced perinuclear localization of MR 30 min after hormone treatment (Fig. 6). Sub-cellular fractionation experiments were performed on M1-CCD cells and M1-CCD cells suppressed in PKD1 expression with and without aldosterone treatment to confirm the role of PKD1 in MR nuclear localization (Fig. 7). Treatment of wild-type cells with aldosterone resulted in an increase in the nuclear abundance of MR within 5 min, which peaked around 30 min and declined to basal levels after 60 min. The redistribution of MR in response to ligand 5 min and 30 min after aldosterone treatment was sensitive to MEK antagonism with PD98059. PKD1-suppression resulted in attenuation of the MR redistribution in response to aldosterone treatment. Taken together, these observations confirmed that the sub-cellular redistribution of MR in response to
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aldosterone, at a time point relevant to the modulation of transcription by aldosterone, was dependent on activation of the MEK/ERK cascade and PKD1 activity.

PKD1 involvement in aldosterone-induced gene expression

MR modulates the expression of many genes in response to aldosterone treatment. The most rapidly detected change in gene transcription following aldosterone treatment is that of SGK-1; where changes in abundance of SGK-1 mRNA can be detected within 20 min, and changes in SGK-1 protein abundance can be detected within 1 h of aldosterone treatment. We previously investigated whether PKD1 suppression could affect the early transcriptional response to aldosterone in the M1-CCD cells, and found that there was no significant effect on the induction of SGK-1 expression by aldosterone after 30 min treatment (21). In this present study we found that aldosterone elicited a diminished increase in ENaC current 4 h after hormone treatment in cells that were suppressed in PKD1 expression as compared to wildtype. Consequently we examined the effect of PKD1 suppression on the induction of SGK-1 protein expression 4 h after aldosterone treatment. We found that SGK-1 expression was barely detectable by Western blotting in both the wild-type and PKD1 suppressed M1-CCD cells without stimulation (Fig. 8A). However, 4 h after aldosterone treatment there was a significant increase in detectable SGK-1 protein. The increase in SGK-1 abundance was 5 to 6-fold in the wild-type cells as compared

to 2-fold in the PKD1 suppressed cells; indicating that the loss of PKD1 expression significantly attenuated the long term effects of MR activation by aldosterone on target gene expression.

The abundance of the ENaCβ subunit is not regulated through transcription by aldosterone in the cells of the distal nephron, and as expected, aldosterone treatment

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did not significantly affect ENaCβ protein abundance in either wild-type or PKD1 suppressed M1-CCD cells (Fig. 8B), in addition the abundance of ENaCβ in the two cell lines before aldosterone treatment also did not differ significantly. PKD1 suppression also did not affect the total abundance of MR in M1-CCD cells, as indicated by Western blotting using an MR-specific MAb (Fig. 8C). This confirmed that the aldosterone-induced effects on MR distribution and SGK-1 expression were a consequence of PKD1 coupled signalling events, and not through non-specific transcriptional effects on total receptor abundance, which would determine the sensitivity of the cells to aldosterone treatment.

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DISCUSSION

Aldosterone plays a key role in regulating whole body electrolyte homeostasis, and the distal nephron is the pre-eminent site for endocrine regulation of Na^+ efflux from the body. Under the influence of circulatory aldosterone levels, Na^+ is reabsorbed at the luminal or apical surface of the ASDN principal epithelial cells from the renal ultra-filtrate through the ENaC and the Na^+/Cl^- co-transporter. The membrane transporters implicated directly and indirectly at each stage of the Na^+ conservation process are regulated by aldosterone at the transcriptional level over a number of hours after treatment with the hormone (1). The abundance of ENaC at the apical surface of the principal cells of the CCD and other sections of the ASDN is the ratelimiting factor in affecting Na^+ re-absorption in the distal nephron. However, a role for the earliest rapid signalling responses stimulated by aldosterone in the CCD in promoting Na^+ transport has been unclear. This is essentially because the detection of increased ENaC activity in cell culture models, through the measurement of I_{SC}, occurs much later than the measurable effect of aldosterone on the ionic composition of the renal ultra-filtrate from *in vivo* experiments would predict (29). The redistribution of pre-expressed ENaC has been linked to changes in SGK-1 expression within 1 h of aldosterone treatment, and the earliest changes in SGK-1 expression using a relatively physiological concentration of aldosterone (10 nM) was 30 min after hormone treatment (30). The earliest changes in ENaC activity described in the literature are detected within 5 min of aldosterone treatment (8, 29), and must depend upon signalling events not coupled to the later detected increase in SGK-1 abundance.

Functional ENaC is a heterotrimer that assembles in the endoplasmic reticulum, where the subunits undergo *N*-linked glycosylation; further posttranslational modification occurs on passage through the Golgi, this includes furin cleavage of the -α and -γ subunits and the concurrent substitution of a simple, high mannose

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glycosylation pattern for a complex one. The glycosylation and proteolytic processing of the ENaC channel are prerequisite for its full activity; however, partially processed or immature ENaC subunits are also associated with the cell membrane (31). Under high Na^+ conditions ENaC is found in vesicles throughout the cytoplasm of CCD cells, and Na^+ depletion or aldosterone exposure result in translocation of the ENaC vesicular pool to the apical cell membrane well in advance of ENaC subunit transcriptional changes (32). Changes in the rate of membrane integration of ENaC may be a mechanism for aldosterone-induced, signal transduction cascades to impact upon ENaC activity, so augmenting the transcriptional effects of MR.

The activation of protein kinase signalling cascades has been implicated in ENaC

activation; PKA regulates Nedd4-2 ubiquitin ligase in response to vasopressin, to promote ENaC activation 5 to 15 min after treatment (33). PKC has been implicated in the direct phosphorylation of each of the ENaC subunits, and also to increase channel activity in response to insulin in A6 cells (34). In apparent contradiction to this effect, the activation of a negative feedback pathway involving PKC and ERK1/2 to suppress ENaC activity through enhanced proteasome degradation of the ENaCγ subunit has been described in the same cell line treated with phorbol ester for >1 h (35). The same group described a signalling crosstalk mechanism involving ERK1/2 and PI3-K pathways following aldosterone treatment; where PI3-K activation promoted ENaC activity through SGK-1, while ERK1/2 activation had a suppressive effect on ENaC activity (36). In this model the activation of ERK1/2 is mediated through elevated expression of its upstream regulator, the small G-protein K-RasA, which is detected 1 h after aldosterone treatment rather than a rapid response within minutes of treatment (36). Thus early ENaC activity is stimulated by aldosterone through the transcriptional regulation of SGK-1 and K-RasA. The K-RasA polypeptide is also methylated in response to aldosterone in A6 cells and activation of ENaC is sensitive to protein methylation blockade (37, 38). There is therefore the potential for

rapid activation of ENaC following Ras activation perhaps through a Raf-1/ERK1/2 pathway.

The endocrine modulation of sub-cellular trafficking is a developing field and the activation of signalling intermediates by aldosterone, such as PKD1 that is involved in vesicular trafficking, may be an important facet of this hormones activity (13). The activation of PKD1 at the TGN results in its persistence at this organelle, rather than shuttling to the nucleus; which is the response generally observed in response to cell membrane-associated PKD1 activation. Through its TGN association, PKD1 has been implicated in modulating intracellular trafficking, by regulating the fission of vesicles from the TGN (16, 18, 39). The precise mechanism for this is unclear; evidence points to significance in the interaction of PKD with the TGN-associated kinase PI-4 kinase III-β (16). The activation of PKD1 in response to aldosterone coincides temporally with early changes in ENaC subunit redistribution that was observed in a previous study (21). Rapid surface translocation and increased ENaC activity has been reported in response to other agonists; for example, forskolin induced a two-fold increase in amiloride sensitive I_{SC} after 25 min of treatment in a CCD cell line, with a simultaneous increase in surface exposure of ENaC subunits (40).

In this present study, aldosterone induced a measurable increase in ENaC activity in the M1-CCD cells 2 to 4 h after hormone treatment that was maximal after 16 to 24 h, and which coincided with the sub-cellular redistribution of ENaCβ. We observed that ENaCα was expressed at higher basal levels in PKD1 suppressed cells than in wildtype cells; ENaCα did not become inserted into the apical membrane after aldosterone treatment, instead ENaCα remained most abundant at the basolateral surface. This indicated that the process of trafficking ENaCα to the apical membrane

was defective in PKD1 suppressed cells. Insertion of ENaC into the apical membrane is prerequisite for its ubiquitination and retrieval to the sub-apical pool, or to the proteasome for degradation. Ubiquitination of ENaC subunits requires the Ca^{2+}-dependent translocation of Nedd4-2 to the apical membrane (41). A failure of PKD1-dependent ENaC trafficking to the apical membrane, therefore results in an increase in total ENaC abundance without an increase in channel activity. The PKD family isoforms have different roles in establishing the structure of the Golgi and regulating the TGN to membrane trafficking; PKD2 and PKD3 kinase-dead mutants caused TGN tubulation and inhibited TGN to membrane transport in a non-polarized cell monolayer. In the same study, PKD2 was implicated in basolateral membranespecific

vesicle trafficking in a polarized epithelium (42).
In addition to the sub-cellular trafficking effects, the activation of PKD1 has also been implicated in the regulation of gene expression, through the phosphorylation of transcription factors and their regulating kinases. PKD1 phosphorylates IKK to promote NFκB-dependent transcription in response to oxidative stress (26), PKD1 phosphorylates cJun to suppress its transcriptional activity in response to EGF (43) and PKD isoforms stabilize ERK1/2 activity to promote cell growth (27, 44). ERK1/2 activation is the most frequently described signalling response to aldosterone (45, 46), and the stabilization of ERK1/2 activation by aldosterone is also PKD1-dependent (47). Oestrogen promotes ERK1/2 activation, and activated ERK1/2 phosphorylates oestrogen receptor at Ser118, to promote its nuclear localization (28). In this present study we found that activation of the ERK1/2 signalling cascade was also necessary to stabilize the association of MR with the cell nucleus following aldosterone treatment, suggesting a mechanism of nuclear localization that is common to diverse nuclear steroid receptors. Suppression of PKD1 expression inhibited the association of MR with the nucleus. This observation demonstrates a role for PKD1 not only in determining the localization of pre-expressed ENaC through
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the regulation of vesicle trafficking, but also promoting aldosterone-stimulated gene expression by stabilizing the association of MR with the nucleus in combination with ERK1/2. The activation of PKD1 by aldosterone is an integration point between the rapid signalling events stimulated by the hormone, and the latent transcriptional events directed by MR. PKD1 is part of a cascade that integrates the cell signalling and transcriptional effects of aldosterone to modulate renal physiology.

Acknowledgement
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FIGURE LEGENDS

Fig. 1. *Aldosterone-induced ENaCα translocation is PKD1-dependent.* Wild-type M1-CCD cells or cells suppressed in PKD1 expression were grown on cover slips until confluent then serum starved for 24 h. Cells were then either left untreated or treated with 10 nM aldosterone for 24 h (A). Cells were fixed using 4% paraformaldehyde. ENaCα was detected by immuno-fluorescence (green) and polymerized actin was stained with TRITC conjugated to phalloidin (red). Cells were visualized using a Zeiss LSM 510 Meta confocal microscope with excitation wavelengths of 488 nm (Alexa488) and 543 nm (TRITC). Images are representative of XY planes at indicated levels through the epithelium. The apical (a) and basolateral (b) surfaces of the epithelium are indicated. ZX planes through the stained monolayer with apical surface indicated (arrow) are also shown (B).

Fig. 2. *Aldosterone-induced ENaCβ apical membrane insertion is PKD1-dependent.* Wild-type M1-CCD cells or cells suppressed in PKD1 expression were grown on cover slips until confluent then serum starved for 24 h. Cells were then either left untreated or treated with 10 nM aldosterone for 24 h. The cell membrane was stained with wheat germ agglutinin conjugated to Alexa546 (red) and cells were fixed using 4% paraformaldehyde. ENaCβ was detected by immunofluorescence using a specific antibody and an Alexa488 conjugated secondary conjugate (green). Cells were visualized using a Zeiss LSM 510 Meta confocal microscope with excitation wavelengths of 488 nm (Alexa488) and 543 nm (Alexa546). Images are representative of YZ plane through the epithelium. Co-localization of ENaCβ with the

apical membrane (yellow) is indicated (arrows).

Fig. 3. *Aldosterone stimulation of the amiloride-sensitive I_{TE} is PKD1-dependent.* Wild-type M1-CCD cells or cells suppressed in PKD1 expression were

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seeded ($1x10^5$ cells per support) onto semi-permeable supports in normal culture medium containing dexamethasone (1 μM). The cells were allowed to grow with culture medium changes every two days and trans-epithelial electrical resistance (R_{TE}) was measured each day (A). Wild-type M1-CCD cells or cells suppressed in PKD1 expression were seeded ($1x10^5$ cells per support) onto semi-permeable supports in normal culture medium containing dexamethasone (1 μM). The cells were allowed to grow with culture medium changes every two days. Once each support had reached high R_{TE} (> 800 Ω) medium was replaced with serum free containing dexamethasone for 6 h. The epithelia were then treated with 10 nM aldosterone or left untreated and the R_{TE} and trans-epithelial potential difference (V_{TE}) were measured at the indicated time points (B). Amiloride was added to determine the contribution of ENaC. The trans-epithelial current (I_{TE}) was calculated using Ohm's law.

Fig. 4. PKD1 suppression and MEK antagonism affects *MR redistribution 2 min after aldosterone treatment.* Wild-type M1-CCD cells and M1-CCD cells suppressed in PKD1 expression were grown on cover slips in growth medium containing serum until fully confluent then grown in serum free medium for 24 h. Cells were then either pretreated with PD98059, treated with 10 nM aldosterone for 2 min or left untreated as indicated. Cells were fixed using 4% paraformaldehyde, the subcellular distribution of MR was determined by immunofluorecence using a MRspecific monoclonal antibody and detected using a murine IgG-specific antibody conjugated to Alexa 488 (green). The cell nuclei were counter stained with DAPI (red). Cells were visualized using a Zeiss LSM 510 Meta confocal microscope with excitation wavelengths of 488 nm (Alexa 488) and 364 nm (DAPI). Images are representative of a single XY focal plain mid-section through the epithelium.

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Fig. 5. PKD1 suppression affects *MR redistribution 30, 60, 120 min after aldosterone treatment.* Wild-type M1-CCD cells and M1-CCD cells suppressed in PKD1 expression were grown on cover slips in growth medium containing serum until fully confluent then grown in serum free medium for 24 h. Cells were then either treated with 10 nM aldosterone for 30, 60 or 120 min or left untreated. Cells were fixed using 4% paraformaldehyde, the sub-cellular distribution of MR was determined by immunofluorecence using a MR specific monoclonal antibody and detected using a murine IgG specific antibody conjugated to Alexa 488 (green). The cell nuclei were counter stained with DAPI (red). Cells were visualized using a Zeiss LSM 510 Meta confocal microscope with excitation wavelengths of 488 nm (Alexa 488) and 364 nm (DAPI). Images are representative of a single XY focal plain mid-section.

Fig. 6. *Aldosterone-induced MR translocation is ERK1/2-dependent at 30 min.* M1-CCD cells were grown on cover slips in growth medium containing serum until fully confluent then grown in serum free medium for 24 h. Cells were pre-treated with the MEK antagonist PD98059 (1 μM). Cells were then either treated or left untreated with 10 nM aldosterone for 30 min. Cells were fixed using 4% paraformaldehyde, the sub-cellular distribution of MR was determined by immunofluorecence using a MR specific monoclonal antibody and detected using a murine IgG specific antibody conjugated to Alexa 488 (green). The cell nuclei were counter stained with DAPI (red). Cells were visualized using a Zeiss LSM 510 Meta confocal microscope with excitation wavelengths of 488 nm (Alexa 488) and 364 nm (DAPI). Images are representative of a single XY focal plain mid-section.

Fig. 7. *PKD1 and ERK1/2 activation promote MR nuclear localization in response to aldosterone.* Nuclear and cytoplasmic fractions were prepared from

M1-CCD cells and cells suppressed in the expression of PKD(-) after the indicated times of aldosterone treatment. Western blots were performed on the sub-cellular
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fractions to determine the re-distribution of MR following treatment. A laminin specific antibody was used to confirm the efficacy of fractionation. The involvement of the MEK/ ERK cascades was determined by pre-incubation with PD98059. The gel images are representative of three independent experiments.

Fig. 8. ***PKD1 involvement in aldosterone-induced gene expression.*** Lysates were prepared from wild-type M1-CCD cells and M1-CCD cells suppressed in PKD1 expression (PKD -) either treated with aldosterone (10 nM) for 4 h (+), vehicle treated for 4 h (-) or not treated (0). Equivalent amounts of protein were separated on a SDSPAGE gel (80 μg protein) then Western blotted and probed using a SGK-1 specific antibody (A) an ENaCβ specific antibody (B) and a MR specific antibody (C). Equal loading was confirmed by re-probing membranes for β-actin expression and the images shown are representative of three independent experiments.
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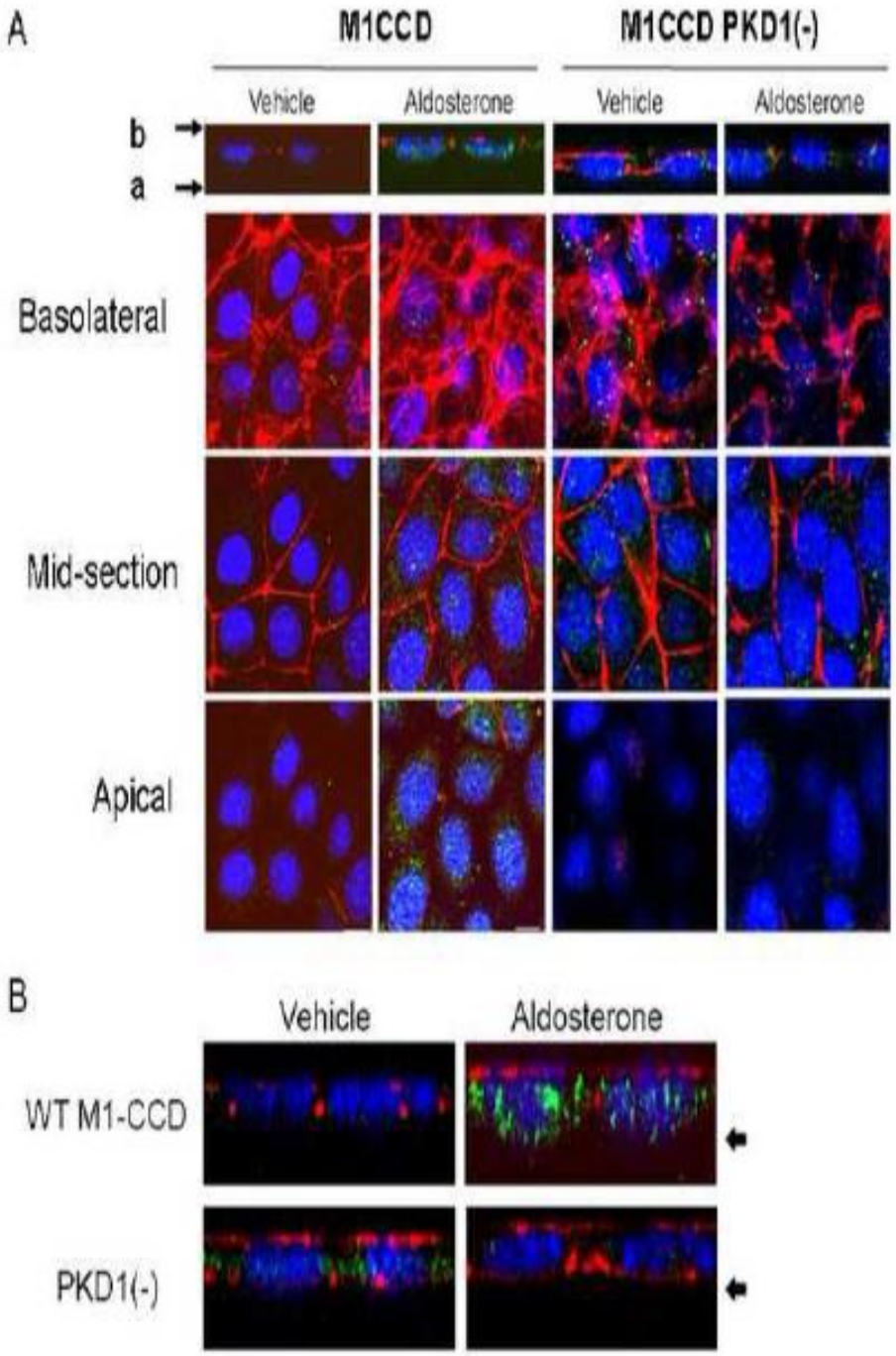
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Fig. 1

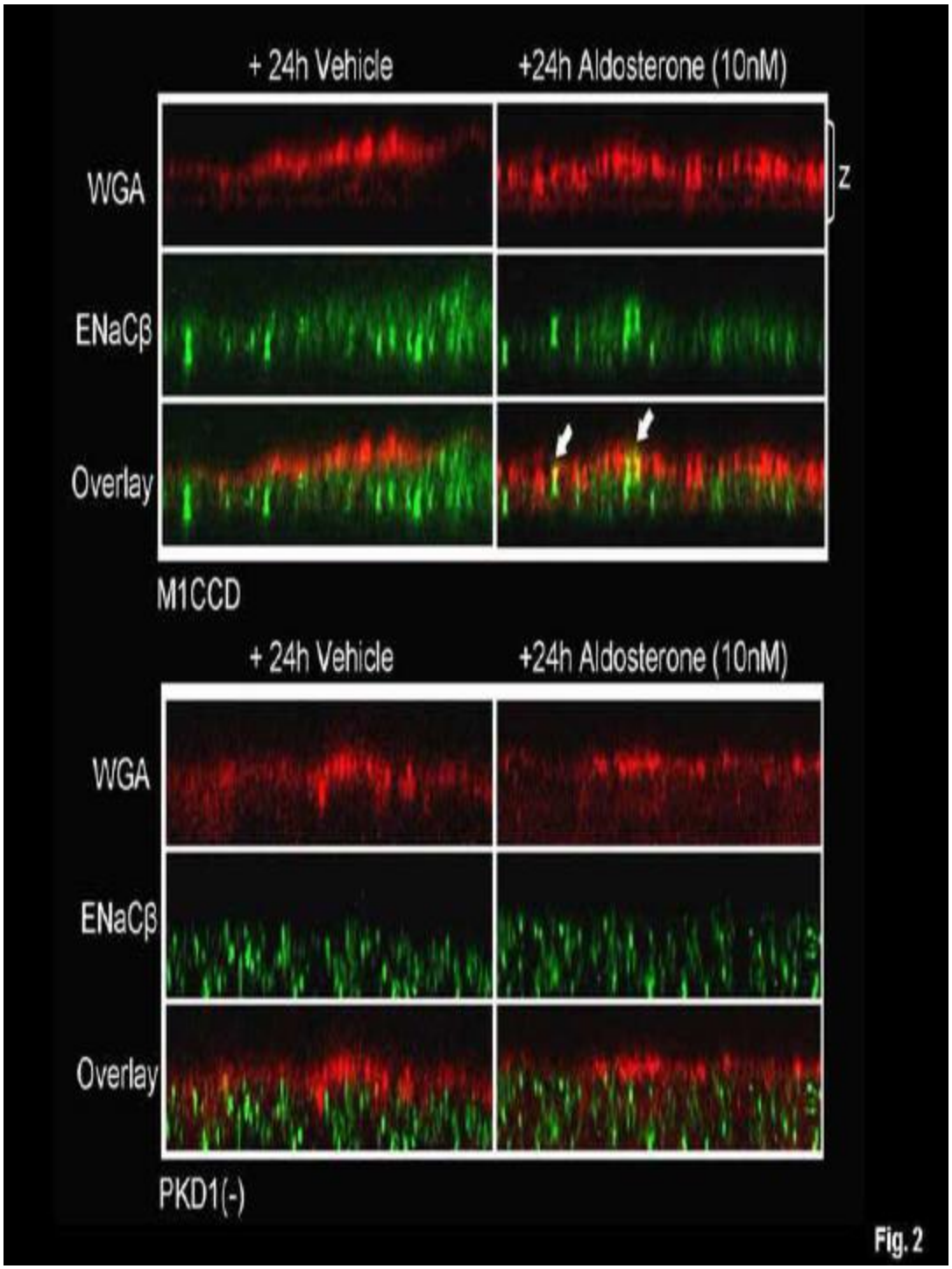
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Fig. 2

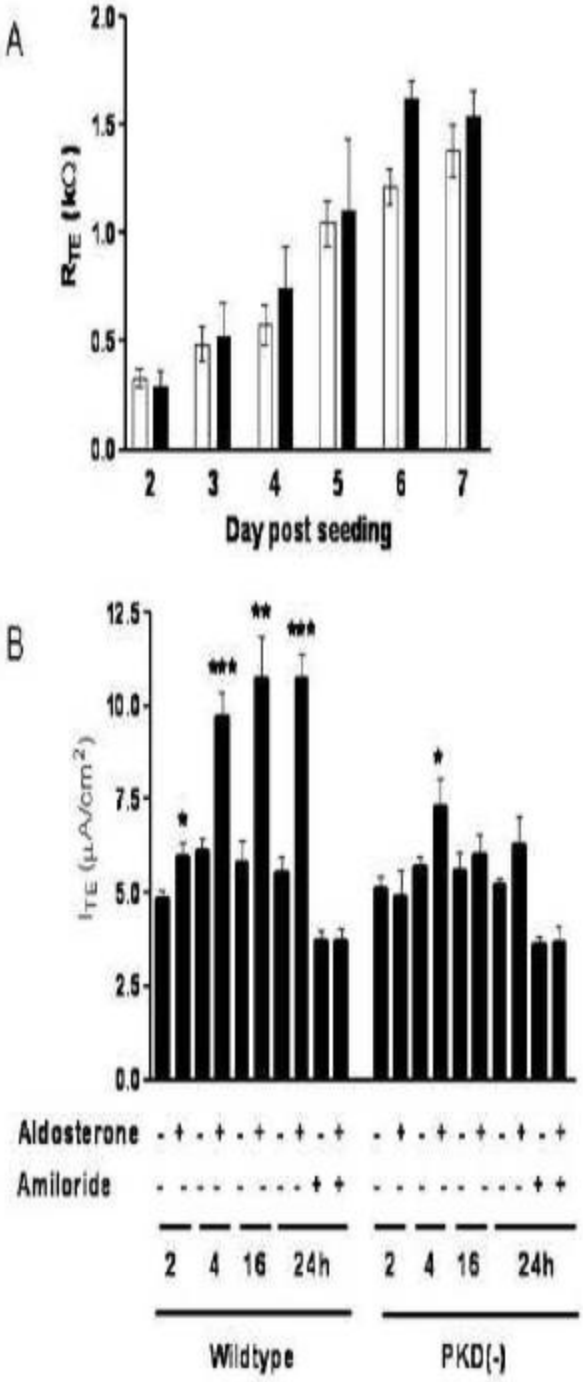
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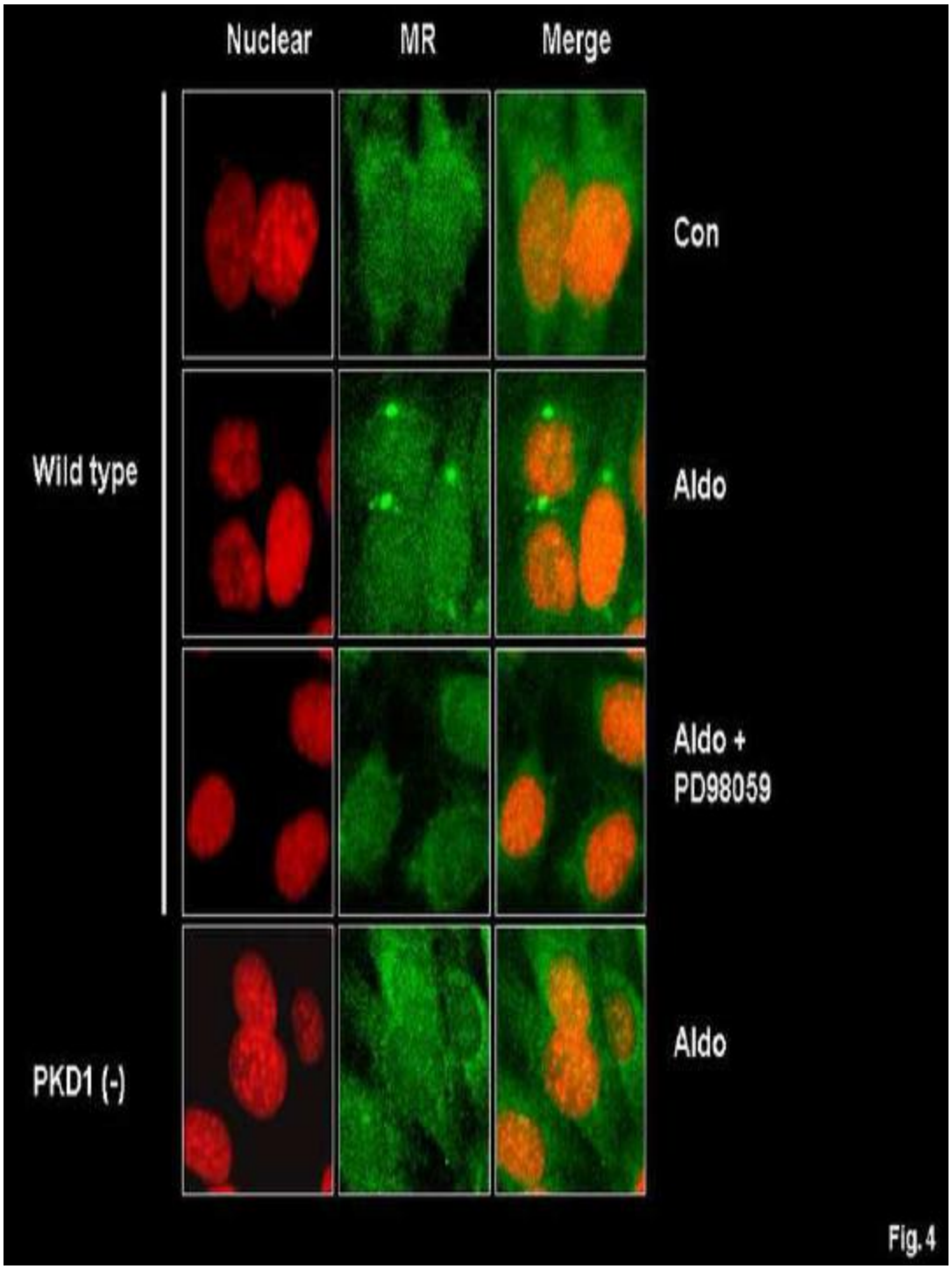
Fig. 3



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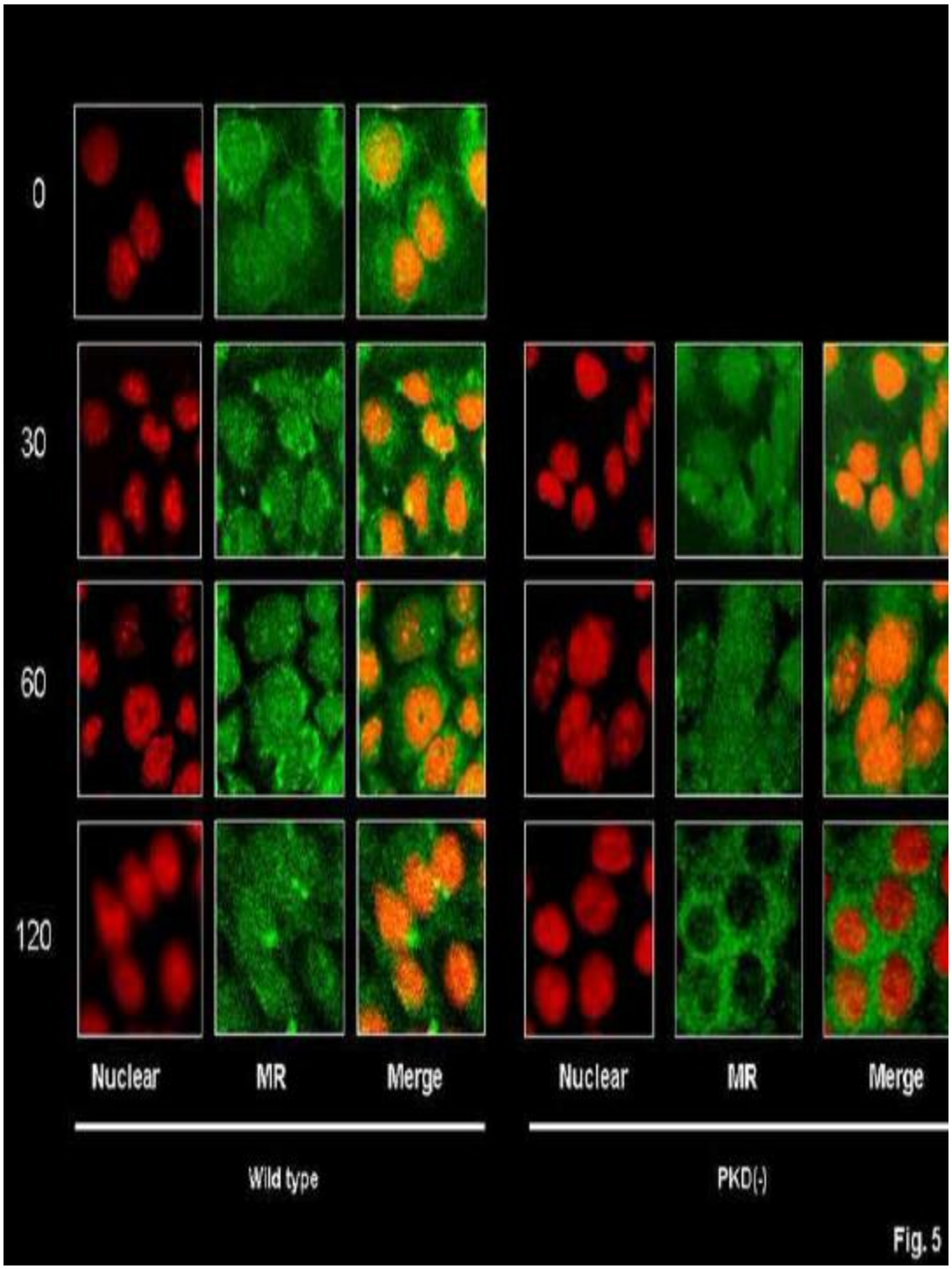

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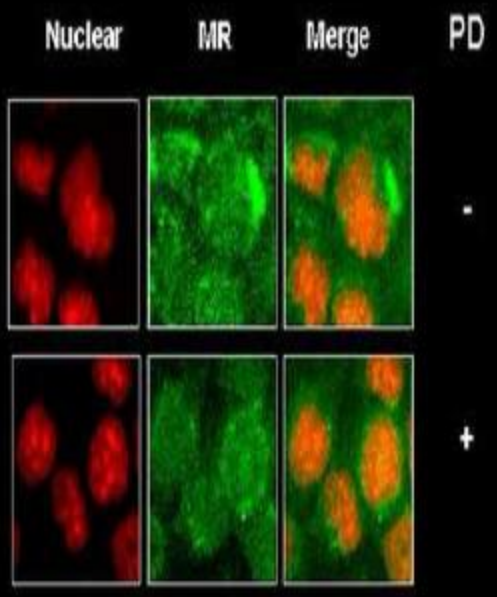

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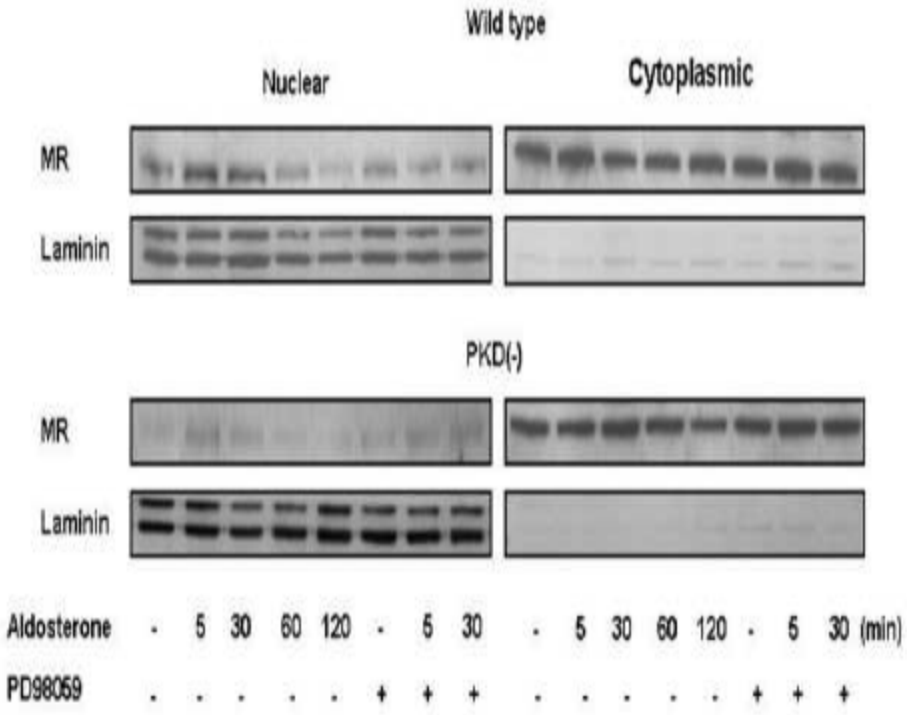
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Fig.7

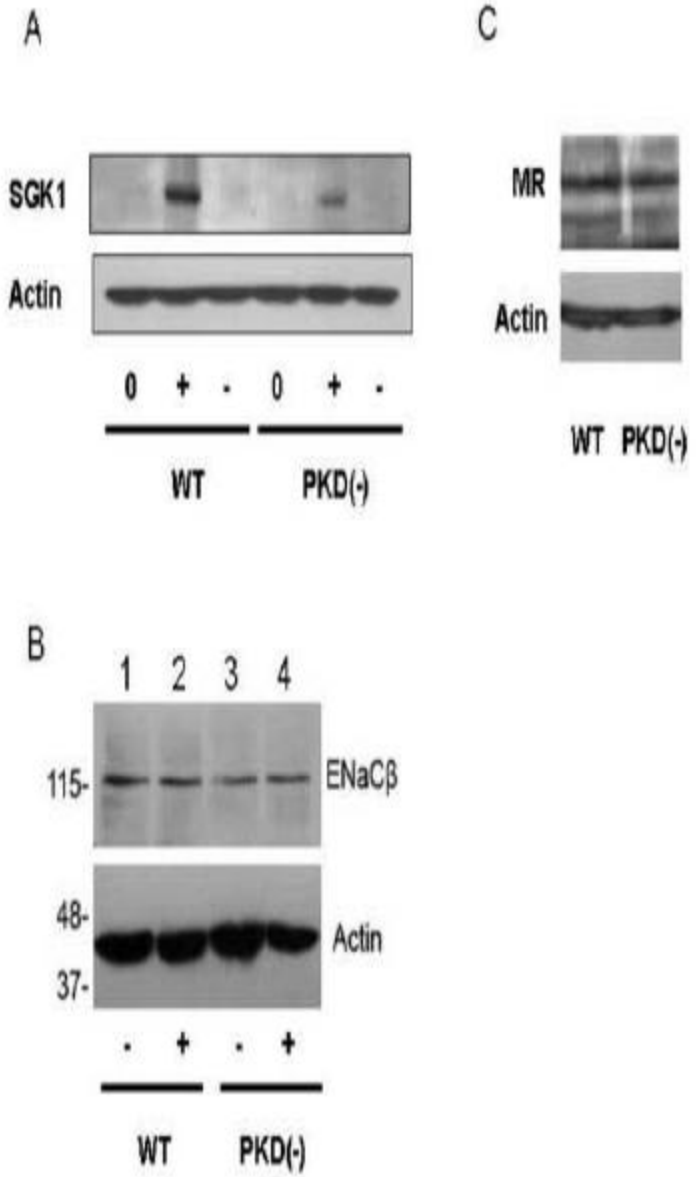
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Fig.8