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Aldosterone-induced ENaC and basal Na^+/K^+-ATPase trafficking via protein kinase D1-phosphatidylinositol 4-kinaseIIIβ trans Golgi signalling in M1 cortical collecting duct cells

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ABSTRACT

Aldosterone regulates Na^+ transport in the distal nephron through multiple mechanisms that include the transcriptional control of epithelial sodium channel (ENaC) and Na^+/K^+-ATPase subunits. Aldosterone also induces the rapid phosphorylation of Protein Kinase D1 (PKD1). PKD isoforms regulate protein trafficking, by the control of vesicle fission from the trans Golgi network (TGN) through activation of phosphatidylinositol 4-kinaseIIIβ (PI4KIIIβ). We report rapid ENaCγ translocation to the plasma membrane after 30 min aldosterone treatment in polarized M1 cortical collecting duct cells, which was significantly impaired in PKD1 shRNA-mediated knockdown cells. In PKD1-deficient cells, the ouabain-sensitive current was significantly reduced and Na^+/K^+-ATPase α and β subunits showed aberrant localization. PKD1 and PI4KIIIβ localize to the TGN, and aldosterone induced an interaction between PKD1 and PI4KIIIβ following aldosterone treatment. This study reveals a novel mechanism for rapid regulation of ENaC and the Na^+/K^+-ATPase, via directed trafficking through PKD1-PI4KIIIβ signalling at the level of the TGN.

1. Introduction

Aldosterone is released from the adrenal glands in response to hyperkalaemia or through hypotension-induced activation of the renin-angiotensin cascade to modulate the physiology of cells that express the mineralocorticoid receptor (MR). Aldosterone contributes to whole body Na^+ homeostasis through the tight control of ion transporters contributing to Na^+ conservation by the cells of the distal nephron. Na^+/K^+-ATPase pump activity at the basolateral membrane of these cells provides the driving force for the reabsorption of Na^+ from the renal ultra-filtrate at the apical membrane through the epithelial sodium channel (ENaC) by maintaining a low intracellular Na^+ concentration. Functional ENaC is presumed to be a heterotrimer of 1α, 1β, and 1γ subunit, based on the resolved crystal structure of the closely related acid-sensing ion channel, ASIC1 (Jasti et al., 2007). The functional Na^+/K^+-ATPase is a heterodimer comprising one catalytic α subunit and one regulatory β subunit. The α subunit has 10 trans-membrane domains and accommodates the binding sites for ATP, Na^+, K^+ and ouabain as well as the catalytic phosphorylation site (Lingrel and Kuntzweiler, 1994). The β subunit is important for proper maturation, delivery and insertion of the αβ heterodimer into the basolateral plasma membrane and influences the catalytic activity of the pump (Jaisser et al., 1994).

Aldosterone regulates the activity of both transporters through multiple mechanisms in order to maintain normal blood Na^+ concentrations within a very narrow range (137–147 mM) (Payne and Levell, 1968). The best studied aspect of aldosterone action is its stimulation of MR as a ligand-dependent transcription factor. Activated MR promotes the expression of ENaCα (Asher et al., 1996), together with the α and β subunits of the Na^+/K^+-ATPase (Kolla and Litwack, 2000). Aldosterone can also regulate the expression of signalling intermediates that modulate transporter abundance such as the serum and glucocorticoid regulated kinase (SGK)-1 (Alvarez de la Rosa et al., 1999; Chen et al., 1999; Naray-Fejes-Toth and Fejes-Toth, 2000). SGK1 phosphorylates and inhibits Nedd4-2, an E3 ubiquitin ligase, so promoting stability of ENaC at the apical membrane (Snyder et al., 2002).

Aldosterone treatment induces rapid signalling effects in multiple tissues including the principal cells of the renal cortical collecting duct (CCD) and these signals are not dependent on *de novo* protein synthesis. We have shown that aldosterone induces the rapid phosphorylation and activation of Protein Kinase D1 (PKD1) through the MR-dependent trans-activation of the epidermal

Abbreviations: PKD, protein kinase D; ENaC, epithelial sodium channel; Na^+/K^+-ATPase, sodium potassium ATPase; PI4KIIIβ, phosphatidylinositol 4-kinaseIIIβ; M1-CCD, M1 cortical collecting duct; MR, mineralocorticoid receptor; TGN, trans Golgi network.

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growth factor receptor in murine M1-CCD cells (McEneaney et al., 2007). The Protein kinase D family is comprised of three members; PKD1, or its human homolog PKCμ (Johannes et al., 1994; Valverde et al., 1994), PKD2 (Sturany et al., 2001) and PKD3 (human PKCν) (Hayashi et al., 1999). PKD isoforms comprise a subgroup of the Ca^{2+}/calmodulin-dependent protein kinase (CAMK) family (Manning et al., 2002). The novel PKC isoforms such as PKCε and PKCη can phosphorylate and activate PKD (Rozengurt et al., 2005), and we have shown previously that PKD1 activation in M1-CCD cells is PKCε-dependent (McEneaney et al., 2008). Lipid kinases are key regulators of sub-cellular trafficking by regulating the release of cargo-carrying vesicles from the trans Golgi network (TGN). PKD can associate with both Phosphatidylinositol (PtdIns) 4-kinase (PI4KIIIβ) and phosphatidylinositol 4-phosphate (PI4P) 5-kinase through its N-terminus (Nishikawa et al., 1998). Moreover, PKD1 phosphorylates PI4KIIIβ at the Golgi to promote vesicle fission and therefore the rate of protein transport to the plasma membrane (Hausser et al., 2005).

In this present study we investigated the contribution of PKD1 activation to the sub-cellular trafficking of ENaC and Na^+/K^+-ATPase subunits in M1-CCD cells. We investigated the signalling mechanism underpinning this action, in particular the interaction between PKD1 and its known substrate phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ).

2. Materials and methods

2.1. Antibodies and reagents

All reagents used in this study were from Sigma–Aldrich unless otherwise specified. Antibodies used were: PKD1 polyclonal rabbit (Cellular Signaling), PI4KIIIβ polyclonal rabbit (Millipore), Trans Golgi Network protein 38 polyclonal goat (Santa Cruz), ENaCγ polyclonal rabbit (Abcam), Na^+/K^+-ATPase α monoclonal mouse (Abcam), Na^+/K^+-ATPase β monoclonal mouse (Abcam), Aquaporin-2 polyclonal goat (Santa Cruz). Secondary conjugated antibodies: chicken anti-rabbit 488 nm, chicken anti-goat 568 nm, goat anti-rabbit 488 nm (Invitrogen), goat anti-rabbit horse-radish peroxidase (Abcam). Reagents: Aldosterone (Steraloids), Plasma membrane marker: wheat-germ agglutinin (WGA) Alexa633 nm (Invitrogen). Reagents used were: actin stain TRITC-phalloidin (Sigma), Lipofectamine (Invitrogen). Inhibitors: LY294002 (Cell Signaling), PIK-93 (Selleck Chemicals), eplerenone (Tocris). The vesicular stomatitis virus glycoprotein (VSVG-GFP) plasmid was provided by Addgene (Addgene plasmid 11912) (Presley et al., 1997).

2.2. Cell culture

The M1-CCD cell line (ATCC, CRL-2038) is derived from renal CCD micro-dissected from a mouse transgenic for the early region of SV40 virus (strain Tg(SV40E) Bri7) (Stoos et al., 1991). The PKD1 knock-down cell line was created in our lab by stably transfecting M1-CCD cells with an shRNA expressing plasmid specific for PKD1 (McEneaney et al., 2008). M1-CCD cells were maintained in 1:1 Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM:F-12) without phenol red, supplemented with foetal bovine serum (5%), L-glutamine (2 mM), penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and dexamethasone (1 μM). Cultures were maintained in an atmosphere of 70% humidity, 5% CO_2 at 37 °C. Cells were propagated on 6.5 mm diameter Millicell Hanging Cell Culture Inserts (1 μm pore), until a polarized monolayer formed (1500–2000 Ω/cm^2), or on glass cover-slips or 10 cm diameter culture dishes as indicated. Before treatment, cells were maintained in serum-free and dexamethasone-free medium overnight. Aldosterone was prepared as a 50 mM stock in ethanol and further diluted in serum-free, dexamethasone-free medium, to a final concentration of 10 nM. Vehicle controls are the equivalent concentration of ethanol diluted in culture medium. For inhibitor experiments, cells were pre-incubated with 250 nM PIK-93, 25 μM LY294002. 1 μM eplerenone, or equivalent DMSO controls, for 30 min prior to aldosterone treatment.

2.3. Co-immunoprecipitation and Western blotting

Following treatment, cells were washed three times in ice cold phosphate buffered saline (PBS) and lysed using NP40 lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Igepal, 0.25% sodium deoxycholate), with freshly added protease (Roche) and phosphatase inhibitors (Sigma), for 10 min on ice. Lysates were centrifuged at 12,000 rpm for 10 min at 4 °C and supernatants incubated with 2 μl anti-PI4KIIIβ overnight at 4 °C with rotation. The supernatants were incubated with pre-washed Protein G Sepharose beads (GE Healthcare) for 1 h 4 °C and washed using 50% NP40 lysis buffer three times. The IgG control was performed by incubating the lysates with an unrelated primary antibody and did not result in any signal (data not shown). Proteins were eluted by the addition of 2× Laemmli buffer (50 mM Tris–HCl, 2% SDS, 100 mM DTT, 0.2% bromophenol blue, 20% glycerol) and heating to 95 °C for 10 min. Equal volumes were loaded on 8% SDS gels and transferred to nitrocellulose membranes. Blots were blocked in 5% BSA/TBS-T and antibodies diluted in 3% BSA/TBS-T. ECL plus (Amersham Biosciences) was used for chemiluminescent detection. Densitometry was performed using GeneSnap software (Synoptics). Blots were stripped in Restore Western blot stripping buffer (Thermo Scientific) for 30 min room temperature and re-probed with bait antibody.

2.4. Immunohistochemistry

Animals were bred and maintained in an environmentally controlled animal facility according to national regulations. Male Sprague-Dawley rats at 3 months old were sacrificed humanely using vaporized isofluorane and cervical dislocation. Kidneys were removed and rinsed briefly in cold PBS before fixation in 4% PFA for 2 h at room temperature. Tissue was cryoprotected in 30% sucrose/PBS overnight at 4 °C and embedded in Tissue Tek mounting medium on dry ice the following day. Kidneys were stored at −80 °C until sectioning with a Leica cryostat. Sections of 5 μm thickness were blocked in 2% gelatin and permeablized in 0.1% Triton X-100. Sections were incubated with antibodies diluted in blocking buffer in a humidified chamber, washed in PBS and embedded in Vectashield Hardset mounting media containing DAPI. Images were acquired using a Zeiss LSM710 laser scanning confocal microscope and a 63× oil objective.

2.5. Immunocytochemistry

Following treatment, cell monolayers were washed twice in cold PBS and fixed at room temperature in 4% PFA/PBS. Cells were blocked and permeabilized in 2% gelatin from cold water fish skin/0.1% TritonX-100/PBS and incubated with primary antibodies as indicated in the figure legends, for 2 h room temperature (RT) followed by secondary antibodies in a dilution of 1:500 for 30 min RT. For experiments using Millicell hanging inserts, semi-permeable membranes were carefully excised from plastic casing, and embedded apical side up under a glass cover-slip, using Vectashield hardest mounting medium containing DAPI. Plasma membrane staining using WGA Alexa633 nm (200 μg ml^{-1} for 5 min) was performed on ice before fixation. TRITC-phalloidin to stain intracellular actin was added in combination with the secondary antibodies at a