1-1-2010

Protein kinase D stabilizes aldosterone-induced ERK1/2 MAP kinase activation in M1 renal cortical collecting duct cells to promote cell proliferation.

Victoria McEneaney
Royal College of Surgeons in Ireland

Ruth Dooley
Royal College of Surgeons in Ireland

Brian J. Harvey
Royal College of Surgeons in Ireland

Warren Thomas
Royal College of Surgeons in Ireland

Citation

McEneaney V, Dooley R, Harvey BJ, Thomas W. Protein kinase D stabilizes aldosterone-induced ERK1/2 MAP kinase activation in M1 renal cortical collecting duct cells to promote cell proliferation. Journal of Steroid Biochemistry and Molecular Biology. 2010;118(1-2):18-28.

Protein Kinase D stabilizes Aldosterone-induced ERK1/2 MAP Kinase Activation in M1 Cortical Collecting Duct Cells to Promote Cell Growth

Victoria McEneaney, Ruth Dooley, Brian J. Harvey and Warren Thomas*

Department of Molecular Medicine,

Royal College of Surgeons in Ireland, Education and Research Centre,

Beaumont Hospital, Dublin 9, Ireland

***Address Correspondence and Reprint Requests to:**

Dr Warren Thomas, Department of Molecular Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland.

Tel. +353 1 809 3825; Fax. +353 1 809 3778; E-mail: wthomas@rcsi.ie

ABSTRACT

Aldosterone elicits transcriptional responses in target tissues and also rapidly stimulates the activation of protein kinase signalling cascades independently of *de novo* protein synthesis. Here we investigated aldosterone-induced cell growth and extra-cellular regulated kinase 1 and 2 (ERK1/2) mitogen activated protein (MAP) kinase signalling in the M1 cortical collecting duct cell line (M1-CCD). Aldosterone promoted the growth of M1-CCD cells, an effect that was protein kinase D1 (PKD1), PKCδ and ERK1/2-dependent. Aldosterone induced the rapid activation of ERK1/2 with peaks of activation at 2 and 10 to 30 min after hormone treatment followed by sustained activation lasting beyond 120 min. M1-CCD cells suppressed in PKD1 expression exhibited only the early, transient peaks in ERK1/2 activation without the sustained phase. Aldosterone stimulated the physical association of PKD1 with ERK1/2 within 2 min of treatment. The mineralocorticoid receptor (MR) antagonist RU28318 inhibited the early and late phases of aldosterone-induced ERK1/2 activation, and also aldosterone-induced cell growth. Aldosterone-induced the sub-cellular redistribution of ERK1/2 to the nuclei at 2 min and to cytoplasmic sites, proximal to the nuclei after 30 min. This sub-cellular distribution of ERK1/2 was inhibited in cells suppressed in the expression of PKD1.

Key Words: Aldosterone, Kidney, PKD1, MAPK, Cell growth.

1. INTRODUCTION

The mineralocorticoid hormone aldosterone is an important modulator of ion fluxes across high resistance epithelia such as that of the colon and the nephron [1]. In the distal nephron, aldosterone action promotes the re-absorption of Na^+ from the renal ultra-filtrate back into the blood and can also stimulate the excretion of excess K^+ from the body through the modulation of ion transporter activity. Aldosterone binds to the mineralocorticoid receptor (MR) in the cytoplasm of target cells to promote its dissociation from accessory proteins and translocation of the receptor into the cell nucleus. In the nucleus MR interacts with the promoter sequences of aldosterone responsive genes to stimulate or suppress their expression [2, 3]. Key target genes of MR include those encoding the subunits of aldosterone-sensitive transporters including the epithelial Na^+ channel (ENaC) and Na^+/K^+ ATPase [4, 5], and also regulators of transporter activity, such as the serum and glucocorticoid regulated kinase (SGK)-1 [6]. In addition to its role as a modulator of ion transport, aldosterone can also stimulate cell growth in target tissues such as in glomerular mesangial cells [7, 8], vascular smooth muscle cells [9] and cardiac fibroblasts [10] with pathological consequences. Aldosterone rapidly activates signalling cascades which promote cell proliferation but the proliferative effects of aldosterone on the renal epithelium is poorly characterised. Non-genomic responses to aldosterone have been described in various target tissues (Reviewed in [11, 12]). These responses are underpinned by the rapid activation of protein kinase signalling cascades. The physiological role of aldosterone-induced signalling is not fully understood however there is definite evidence of rapid aldosterone effects on K^+ channels [13] and Na^+/H^+ exchanger (NHE) activity in renal cells [14, 15]. The transient stimulation of NHE1 activity by aldosterone in cells of the distal nephron is coupled to the rapid activation of the extra-cellular regulated kinase (ERK) 1/2 cascade [16].

ERK1/2 activation is associated with the stimulation of cell growth in response to treatment with growth factors, hormones and other agonists in diverse tissues. Sustained rather than transient activation of the ERK1/2 cascade is required for the stimulation of cell cycle progression by such agonists [17]. This reflects the need for advancement through regulatory checkpoints in the cell cycle and the initiation of transcriptional events. The promotion of cell proliferation by aldosterone has been linked to the rapid activation of the ERK1/2 cascade in mesangial cells [7]. The modulation of ERK1/2 activity has also been

implicated in the regulation of Na^+ re-absorption by cells of the renal cortical collecting duct (CCD) by affecting Na^+/K^+ ATPase activity [18]. The stimulation of cell growth in response to G-protein coupled receptor (GPCR) agonists is dependent on the simultaneous activation of ERK1/2 and protein kinase D1 (PKD1) [17]. The PKD family of serine threonine protein kinases have been implicated in crucial cellular processes including sub-cellular trafficking and cell proliferation (Reviewed in [19]). The activation of PKD by aldosterone may contribute to the proliferative effects of the hormone in renal tissues. Aldosterone promotes the hypertrophy of cardiac myocytes through a PKD1-dependent mechanism that requires sustained activation of PKD1 to modulate gene expression [20]. The activation of ERK1/2 signalling by aldosterone in cells derived from the distal nephron has been described by several groups including our own [21, 22] and this response has been linked to *trans*-activation of the epidermal growth factor receptor (EGFR) [23]. The aberrant stimulation of proliferation in the distal nephron is one factor in the development of polycystic kidney disease [24]. Individuals may carry a genetic predisposition to developing autosomal recessive polycystic kidney disease (ARPKD) and autosomal dominant polycystic kidney disease (ADPKD) through mutations in the genes encoding the fibrocystin or polycystin 1 and 2 genes respectively [25, 26]. Chronic hypertension is an exasperating factor in disease progression [27] and controlling hypertension through the administration of MR antagonists is a potential means for ameliorating the condition [28]. Attenuating the effects of aldosterone on renal epithelial cell proliferation may also have positive consequences by slowing cyst growth and so mitigating the disruption of renal function associated with cyst enlargement.

The M1-CCD cell line is a widely used model for Na^+ transport in the distal nephron. The cells used for growth assays in this investigation were sub-confluent in order to maximise any growth stimulatory effect of aldosterone without complications of contact inhibition. The aim of future work will be to investigate the effects of aldosterone in epithelial monolayers and monolayers of cells with polycystin gene mutations to determine a role for aldosterone in the proliferation observed in polycystic kidney disease. M1-CCD cells polarize and form tight junctions when grown on glass or solid plastic surfaces [29]. Cells grown under these conditions will form a sufficiently polarized and robust epithelium to transport Na^+ directionally with the formation of epithelial domes or blisters. We have observed that the Na^+/K^+-ATPase becomes

localized to the basolateral membrane of the M1-CCD cells when grown on glass [30] and that ENaC becomes localized to the apical membrane (manuscript in preparation). Studies have also been conducted to compare signalling responses on cells grown on non-porous plastic with those grown on semi-permeable supports and it was found that for example the aldosterone-induced activation of c-Src tyrosine kinase was equivalent in M1-CCD cells grown on these two surfaces [31].

We have demonstrated that aldosterone stimulated the activation of PKD in a cortical collecting duct (CCD) cell line through the *trans*-activation of EGFR [32]. In this study we investigate a novel role for PKD1 in MR-dependent, aldosterone-induced cell growth in CCD cells through the modulation of ERK1/2 activity.

2. MATERIALS AND METHODS

2.1 Cell Culture

The M1 cell line [33] is derived from the CCD micro-dissected from a mouse transgenic for the early region of SV40 virus (strain SV40Ebri7). Cells were grown in 75 cm^2 polystyrene culture flasks containing a 1:1 Dulbecco's modified Eagle's medium and Ham's F-12 medium without phenol red supplemented with dexamethasone (5 μM), L-glutamine (2 mM), penicillin (100 $U.ml^{-1}$), streptomycin (100 $\mu g.ml^{-1}$) and foetal bovine serum (10%). The M1-CCD derived cell line stably suppressed in the expression of PKD1 was described previously [30]. M1-CCD PKD1 suppressed cell line culture medium also contained the aminoglycoside antibiotic G418 (200 $\mu g.ml^{-1}$), which is toxic for eukaryotic cells not harbouring a plasmid containing the neomycin resistance gene and is used for selection and maintenance of stably transfected cell lines. Cells were grown in an atmosphere of 70% humidity, 5% CO_2 at 37^oC.

2.2 Preparation of Cell Lysates

For Western blot and immunoprecipitation assays, wild type M1-CCD and PKD knockdown M1-CCD cells were plated in 10 cm dishes, grown to 80% confluency and a quiescent state was induced through growth in serum and dexamethasone deficient medium for 24 h. Cells were incubated with aldosterone (10 nM) or vehicle control for periods indicated. The vehicle control was ethanol diluted $1:10^6$ in serum free DMEM-F12 medium. Following treatment confluent monolayers in 10 cm dishes were washed three times with ice cold

phosphate buffered saline (PBS) and collected in 1 ml PBS in 1.5 ml centrifuge tubes. Lysates were centrifuged for 10 min at 4000 rpm and supernatant removed. The cell pellet was lysed with the addition of 100 μl of lysis buffer (20 mM Tris-HCl pH 7.6, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 1 mM DTT, 10 $\mu g.ml^{-1}$ aprotinin, 10 $\mu g.ml^{-1}$ leupeptin, 10 mM NaF, 1 mM PMSF and 0.5% NP40) at the time points indicated. The protein concentration of the lysates was determined and standardized by Bradford Assay (Bio-Rad).

2.3 SDS-PAGE and Western Blotting

Cell lysates were cleared by centrifugation then dissolved in Laemmli sample buffer. Proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels unless otherwise indicated. The proteins were transferred to PVDF membranes and probed with the specified primary antibodies diluted in 5% bovine serum albumin (BSA) in Tris buffered saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl) according to the supplier's instructions. Bound antibodies were detected using anti-mouse or anti-rabbit horseradish peroxidase secondary antibody conjugates. Labelled bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences). Exposed films were digitally photographed and subjected to densitometric analysis using GeneSnap software (Synoptics).

2.4 Immuno-precipitations

The formation of stable complexes between PKD1 and the ERK1/2 isoforms was determined by co-immunoprecipitation. Cleared lysates were prepared as above in lysis buffer without DTT and incubated with the PKD1 primary antibody. Immuno-complexes were recovered by incubation with protein G sepharose (Amersham Biosciences) and isolated by centrifugation. The immuno-complexes were washed six times with lysis buffer without DTT then subjected to SDS-PAGE and Western blotting as described.

2.5 Cell Growth Assays

The effect of aldosterone and antagonist treatment on cell growth, were measured using a CellTiter 96 MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide) Cell Proliferation Assay (Promega). Sterile 96-

well cell culture plates were equilibrated in a humidified, 5% CO_2 atmosphere. Cells were detached by trypsinization and counted using a Neubauer chamber. Cells were re-suspended to a final concentration of 1 x 10^5 cells.ml^{-1} in cell culture medium with serum. The cells were dispensed (100 μl or 1 x 10^4 cells) into each well of the 96-well plate. The cells were left overnight to adhere and then treated with aldosterone or vehicle control for 48 h. Cells were pre-treated with antagonists for the times indicated. Following treatment, 15 µl of tetrazolium dye solution was added to each well and the plate was incubated for 4 h. Post incubation, 100 µl of solubilisation solution was added to each well for 1 h. The contents of the well were mixed on a shaker in order to achieve a uniform solution. The conversion of the MTT tetrazolium substrate to the formazan product by viable cells was determined by measuring the absorbance at 570 nm using a Multiscan EX plate reader (Thermo).

2.6 Apoptosis Assays

The effect of aldosterone on the incidence of apoptosis occurring in M1-CCD wild-type versus PKD1 suppressed cells was investigated using the Cell Death Detection Assay ELISA plus (Roche Diagnostics GmbH, Germany). This assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, allowing the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. M1-CCD cells and M1-CCD PKD1 suppressed cells were trypsinized and seeded in 96 well plates at a density of 1 x 10^4 cells per well and left to adhere overnight at 37°C, 5% CO_2. The cells were then serum starved for 24 h, before being treated with 10 nM aldosterone or a vehicle control, for 48 h, in the presence of 1 µM dexamethasone and in the absence of serum. Following treatment, the cells were lysed and the intact nuclei pelleted by centrifugation. An aliquot of supernatant was placed in a strepavidin-coated micro-plate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. After removal of unbound antibodies, the amount of peroxidise retained in the immuno-complex was photometrically determined by adding substrate and measuring the absorbance at 405 nm. Each treatment was carried out in duplicate and the experiment was repeated twice. The enrichment factor was calculated by dividing the background corrected absorbance value at 405 nm for aldosterone-treated cells by that for vehicle-treated control cells.

2.7 Immunofluorescence and Confocal Microscopy

M1-CCD cells were grown in 8-well chamber slides (Nunc) until a confluent monolayer was established. Cells were subjected to the treatments indicated then 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. Non specific binding of antibodies and fluorescent conjugates were blocked by addition of 3% BSA in PBS. Bound antibodies (Total ERK1/2 1:400) were detected using goat anti-rabbit Alexafluor 488 conjugate (1:800) (Invitrogen). Cells were mounted in Vectashield (Vector Laboratories) containing 4', 6-diamidino-2-phenylindole (DAPI) and examined using a LSM 510 Meta confocal microscope (Zeiss). Excitation wave[34]lengths for Alexafluor 488 and DAPI were 488 nm and 364 nm respectively. Images were captured at x 63 magnification and x4 zoom. Scans were performed at 1 μm interval depths through the fixed cells and single or merged images are presented as XY single planes through the centre of the cells. Mean nuclear fluorescence intensity values were obtained using Image J software (Rasband, W.S., Image J, NIH, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008). Three random lines were drawn through the nucleus of each cell and the mean fluorescence intensity along each line calculated. The mean fluorescence intensity for the nucleus of each cell was calculated using these three values. In total ~38 cells were quantified for each condition, from four independent experiments. The mean for each condition was plotted as a bar chart ± SEM. Statistical significance was examined using a student's *t* test.

2.8 Antibodies and Antagonists

Antibodies were obtained from Cell Signaling Technology (Total ERK1/2, Phospho-Thr202/Tyr204 ERK1/2, Total PKD1, Phospho-PKD1 Ser916); or from Sigma-Aldrich (β-actin, anti-mouse IgG horseradish peroxidase secondary conjugate and anti-rabbit IgG horseradish peroxidase secondary conjugate). The cells were treated with the following pharmacological antagonists at the concentrations indicated before addition of the hormone. Spironolactone for the specific inhibition of MR (10 μM), Tyrphostin AG1478 for the inhibition of the EGFR (1 μM) and rottlerin (20 μM) for the inhibition of PKCδ and PD98059 (1 μM) for ERK1/2 cascade inhibition were obtained from Calbiochem. RU28318 (10 μM) for the inhibition of MR was obtained from Tocris. Aldosterone was obtained from Steraloids.

2.9 Statistical Analysis

Densitometric values were presented graphically as the means of at least three independent experiments +SEM with one representative Western blot. The analysis of variance (ANOVA) of densitometry values relative to untreated controls was performed using Dunnett's modified t-test on one-way ANOVA data, $p < 0.05$ was treated as significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. RESULTS

3.1 Aldosterone induces growth in M1-CCD cells

Cell signalling cascades rapidly stimulated by aldosterone in the epithelial cells of the distal nephron result in the activation of protein kinases that are implicated in cell growth, these include PKCα [35] and ERK1/2 [16]. Aldosterone contributes to vascular smooth muscle and cardiac myocyte proliferation but the proliferative effects of aldosterone on the renal epithelium is poorly characterised. Aberrant epithelial proliferation is an important factor in the development of polycystic kidney disease [24]. Cyst initiation occurs more frequently in the aldosterone sensitive distal nephron (ASDN) rather than in the proximal nephron where MR is not expressed [36]. The M1-CCD cell line is a widely used model for the ASDN and the physiological effects of aldosterone at this site. The effect of aldosterone treatment on M1-CCD cell growth was measured using the MTT assay. Measurements were performed on both wild-type M1-CCD and M1-CCD cells suppressed in the expression of PKD1 (Fig. 1A). Aldosterone induced a 2 to 3-fold increase in cell growth after 48 h treatment relative to untreated or vehicle treated cells. Aldosterone did not induce an increase in cell number of M1-CCD cells that were suppressed in PKD1 expression relative to vehicle treated cells. This indicates a novel role for PKD1 in promoting aldosterone-induced cell growth in epithelial cells of the CCD. The activation of PKD1 by aldosterone is PKCδ-dependent in M1-CCD cells [30]. Pre-treatment with the PKCδ inhibitor, rottlerin (20 μM) for 30 min completely blocked aldosterone induced proliferation (Fig. 1B) implicating the activation of PKD1 by PKCδ in cell growth. ERK1/2 is rapidly activated following aldosterone treatment of M1-CCD cells. A role for ERK1/2 in mediating cellular proliferation was investigated using the specific ERK1/2 cascade antagonist PD98059. Pre-treatment of M1-CCD cells with PD98059 (1 μM) for 30 min completely blocked aldosterone-induced cellular proliferation (Fig. 1C). The activation of ERK1/2 is thus necessary for aldosterone-induced proliferation in the CCD cells.

The Cell Death Detection ELISA plus assay was used to confirm that the effect of aldosterone on cell growth was due to a stimulation of proliferation rather than the suppression of apoptosis (Fig. 1D). Cytoplasmic nucleosome enrichment was quantified as an indicator of the incidence of apoptosis occurring in wild-type and PKD1 suppressed cell lines with and without aldosterone treatment. We found no statistically significant difference between the amount of apoptosis occurring in M1-CCD wild-type cells and M1-CCD PKD1 knockdown cells treated with 10 nM aldosterone for 48 h. These results support the finding that the increase in cell number in M1-CCD cells after aldosterone treatment is indeed due to an increase in proliferation and that the absence of an increase in cell number in PKD1-deficient cells upon aldosterone treatment is due to an impaired induction of cell proliferation rather than an increased rate of apoptosis.

3.2 Aldosterone induces the cyclical activation of ERK1/2

The activation of ERK1/2 MAP kinase is one of the most widely documented rapid signalling responses to aldosterone and regulates physiological effects such as cytosolic alkalinisation through NHE activation [16] and also aldosterone dependent cell proliferation through the modulation of transcriptional responses [8]. The activation of ERK1/2 has previously been documented in M1-CCD cells. In the present study M1-CCD cells were treated with aldosterone over a time course of 120 min and phosphorylation of ERK1/2 MAP kinases at residues Thr202 and Tyr204 was measured (Fig. 2A). The activation of ERK1 and ERK2 is differentially regulated in response to certain agonists, however, data presented here suggests that both isoforms become equivalently phosphorylated in response to aldosterone, as histograms quantifying ERK1 phosphorylation kinetics mirrored those of ERK2 phosphorylation. ERK1/2 became transiently phosphorylated 2 min after aldosterone treatment and a second peak was observed 10-20 min after aldosterone treatment. A third, sustained phase of ERK1/2 activation was observed that persisted at least until 120 min after aldosterone treatment. Treatment of the cells with an ethanol vehicle control did not elicit a response at any point over this time course. Sustained activation of ERK1/2 by GPCR agonists requires co-activation of PKD1. The activation of ERK1/2 by aldosterone was investigated in M1-CCD cells suppressed in the expression of PKD1 (Fig. 2B). Aldosterone induced the earlier phases of ERK1/2 activation after 2 min and 10-20 min treatment in the PKD1 suppressed M1-CCD cells. The later phase of

ERK1/2 phosphorylation at 90-120 min after aldosterone treatment was not detected in the PKD1 suppressed M1-CCD cells. Sustained activation of ERK1/2 by aldosterone in the M1-CCD cells is therefore dependent on the expression and concurrent activation of PKD1.

3.3 Activation of PKD1 by aldosterone promotes its physical association with ERK1/2

The PKCδ-specific antagonist rottlerin, blocks the aldosterone-induced activation of PKD1 in M1-CCD cells 5 min after treatment with the hormone [30]. Here we show that rottlerin pre-treatment also blocked ERK1/2 activation 2 min after aldosterone treatment (Fig. 3A). The activation of PKD1 and ERK1/2 are both coupled to nPKC activation, however, only the sustained activation of ERK1/2 requires PKD1 co-activation demonstrating a divergence in the signalling cascade at PKCδ. We have previously demonstrated that aldosterone stimulates the rapid, biphasic activation of PKD1 in the M1-CCD cell line [32]. PKD1 forms stable interactions with other protein kinases to facilitate its activation [30] and also forms stable interactions with its substrates once activated. Since PKD1 activation is a pre-requisite for sustained ERK1/2 activation, the establishment of a stable interaction between these two kinases following aldosterone treatment was investigated. Co-immunoprecipitation experiments were performed using a PKD1-specific polyclonal antibody. The abundance of ERK1/2 associated with PKD1 was determined before and after aldosterone treatment (Fig. 3B). The immunoprecipitated proteins were separated by SDS-PAGE, Western blotted and probed with a total ERK1/2 MAP kinase antibody and a total PKD1 specific antibody. ERK1/2 was present in complex with PKD1 without aldosterone treatment, however, treatment with the hormone promoted the formation of a stable complex between PKD1 and ERK1/2 MAP kinase and the abundance of ERK1/2 associated with PKD1 increased 8-fold after 2 min hormone treatment as compared to untreated cells. The increased physical interaction between PKD1 and ERK1/2 confirmed a role for PKD1 activation in modulating aldosterone induced ERK1/2 activity.

3.4 Aldosterone-induced cell growth is dependent on MR and EGFR

The interaction of aldosterone with MR facilitates the role of the receptor as a ligand-dependent transcription factor by promoting its nuclear translocation. The role of MR as the initiator of non-genomic signalling processes has been the subject of some debate, however the stimulation of at least some of the

non-genomic signalling events is MR-dependent. The stimulation of ERK1/2 activation in mesangial cells by aldosterone was sensitive to MR antagonism, which also blocked aldosterone induced cell proliferation [7]. The involvement of MR in the stimulation of cell growth by aldosterone in the M1-CCD cells was investigated using the specific and widely used MR antagonist, spironolactone. Pre-incubation of M1-CCD cells with spironolactone (10 μM) for 30 min prior to aldosterone treatment blocked the stimulation of increased cell growth seen after 48 h treatment with the hormone (Fig. 4A). The activation of PKD1 and ERK1/2 by aldosterone in the M1-CCD cells is dependent on the *trans*-activation of EGFR. EGFR activation promotes cell growth and EGF antagonism decreased the growth activity in cysts from a murine polycystic kidney disease model [37]. The involvement of EGFR *trans*-activation in aldosterone-induced M1-CCD cell growth was investigated using the EGFR antagonist tyrphostin AG1478. Pre-incubation of the cells with AG1478 blocked the stimulation of cell growth by aldosterone over the subsequent 48 h (Fig. 4B). The stimulation of cell growth by aldosterone in the M1-CCD cells therefore relies upon the interaction of aldosterone with the classical MR and subsequent *trans*-activation of EGFR.

3.5 Activation of ERK1/2 by aldosterone is mediated by MR

Different, specific antagonists of MR have produced contradictory data in their attenuation of the non-genomic, signalling responses stimulated by aldosterone. This includes differing effects in the inhibition of the ERK1/2 cascade (reviewed in [11, 12]). These differential effects may be attributed to the differences in structural flexibility of open versus closed E-ring structures within the antagonists. The effectiveness of four structurally distinct MR antagonists on ERK1/2 activation has been reported in the M1-CCD cell line with 10 min aldosterone treatment. The authors reported on the inefficacy of the antagonists to blunt the aldosterone induced ERK1/2 response at this time-point, however, some inhibition of the ERK1/2 response was evident in the presence of canrenoate and RU28318 [22]. Activation of ERK1/2 in our experimental system peaked at 2 and 120 min after aldosterone treatment (Fig 2). The effect of RU28318 on aldosterone-induced ERK1/2 activation at these time points was investigated here. The stimulation of ERK1/2 activation by aldosterone in M1-CCD cells after 2 and 120 min treatment was blocked by pre-incubation with the MR antagonist (Fig. 5). The Western blot was re-probed with a total ERK1/2 rabbit polyclonal antibody to demonstrate that there was no significant change in ERK1/2 abundance over the

course of this experiment. The activation of ERK1/2 during the early transient phase and later sustained phase is thus dependent on aldosterone binding to the classical MR.

3.6 Aldosterone induces PKD1-dependent redistribution of ERK1/2

The activation of ERK1/2 in response to mitogens is associated with its translocation from the cytoplasm to the nucleus [38], where it participates in the phosphorylation of growth promoting transcription factors such as Elk-1 (reviewed in [39]). The stimulation of ERK1/2 phosphorylation in the M1-CCD cells by aldosterone was detectable by Western blotting 2 min after hormone treatment (Fig. 2). Changes in the sub-cellular localization of ERK1/2 following aldosterone treatment were investigated by confocal immunofluorescence microscopy. In untreated wild-type M1-CCD cells, ERK1/2 was uniformly distributed throughout the cytoplasm with some nuclear localization (Fig. 6A). Following aldosterone treatment ERK1/2 became localized to the nuclei of the cells and quantification of the immunofluorescent staining in the nuclei of single focal plane scans indicated that there was a two-fold increase in fluorescence in the Alexa 488 emission range 2 min after aldosterone treatment. In PKD1 suppressed M1-CCD cells, ERK1/2 was largely localized to the cell cytoplasm, again with some ERK1/2 staining evident in the nucleus (Fig. 6B). In these cells, aldosterone treatment did not stimulate the redistribution of ERK1/2 to the nuclei as observed in the wild-type cells even though the transient ERK1/2 phosphorylation at the time point as indicated by Western blotting, was comparable in wild-type and PKD1 suppressed cells (Fig. 2). After 30 min aldosterone treatment, ERK1/2 localized to the nuclei or to discrete cytoplasmic sites proximal to the nuclei in wild-type cells. In PKD1 suppressed cells there was no change in the distribution of ERK1/2, which remained largely in the cytoplasm (Fig.7). The translocation of ERK1/2 into the nucleus and subsequently to discrete cytoplasmic sites in response to aldosterone is thus dependent on the concurrent activation and association with PKD1.

Aldosterone treatment of wild-type cells resulted in a sustained phase of ERK1/2 phosphorylation that was detectable for at least 120 min after treatment. This sustained phase of ERK1/2 activation coincided with the accumulation of the kinases at discrete sites in the cytoplasm of wild-type cells, proximal to the nuclei with much diminished abundance of the kinases within the nuclei (Fig. 8). Aldosterone treatment of PKD1 suppressed cells for 120 min did not stimulate a sustained phase of ERK1/2 phosphorylation and ERK1/2

remained evenly distributed throughout the cytoplasm similar to that observed after 2 min aldosterone treatment. Aldosterone stimulates the sub-cellular redistribution of ERK1/2 to the nuclei within 2 min of treatment with a further change in localization to discrete cytoplasmic sites coinciding with its sustained activation. Suppression of PKD1 expression attenuated the redistribution of ERK1/2 in response to aldosterone over both short and long-term phases of activation. The association of ERK1/2 with the nucleus is transient [40] and the export of ERK1/2 back to the cytoplasm is due to dephosphorylation by nuclear phosphatases or the nucleus to cytoplasm shuttling of MEK [41].

4. DISCUSSION

The modulation of renal ion transport by aldosterone is well studied and defines a mechanism where the action of aldosterone on the kidney can modulate whole body electrolyte balance. The pathophysiological consequences of excessive stimulation of aldosterone-dependent processes, particularly Na^+ re-absorption include hypertension with resulting cardiovascular and renal damage. The promotion of hypertension is itself a mechanism by which aldosterone can affect the structure of the renal tubule [42, 43]. Spironolactone augments the effects of Angiotensin converting enzyme (ACE) inhibitors in reducing urinary protein excretion in chronic kidney failure [43]. ERK1/2 activation has been implicated in aldosterone induced renal damage in a hypertensive rat model [44]. The contribution of elevated plasma aldosterone levels to renal disease through the direct action of steroid hormones on modulating kidney tubule differentiation and proliferation is less well documented. Mesangial cell proliferation is a contributory factor in the development of glomerularnephritis and aldosterone promotes proliferation in cells of the mesangial epithelium through a mechanism involving the ERK1/2-dependent stimulation of cyclin D1 and cyclin A expression [8]. The aldosterone-induced stimulation of cell growth also plays a role in normal renal development. Aldosterone has been specifically implicated in the stimulation of cell proliferation and differentiation in the developing renal tubule [45]. The *in vitro* development of renal tubules from isolated embryonic stem cells can only be achieved in the presence of aldosterone, an effect that is MR-dependent and not mimicked by glucocorticoids or other steroid hormones [45].

Aldosterone-induced ERK1/2 activation in CCD-derived cells is coupled to the activation of NHE1 [21] resulting in cytoplasmic alkalinization. A transient rise in intracellular pH is variously described as a prerequisite or at least permissive for the initiation of cell cycle progression in response to growth factors [46, 47] and may be synergistic with the transcriptional events stimulated by ERK1/2 activation to promote cell growth. ERK1/2 activation reported in our previous study was biphasic over a 30 min time course with peaks 2 min and 20 min after hormone treatment [21]. Aldosterone stimulates activation of ERK1/2 in amphibian A6 renal cells with a peak 1 to 2 h after treatment [48]. However, it has been shown that A6 cells lack MR and that aldosterone stimulated processes are initiated at the glucocorticoid receptor (GR) [49]. The activation of ERK1/2 in the A6 cells was sensitive to GR antagonism and relied on transcriptional up regulation of Ki-RasA [48].

Our present study demonstrates that in MR-expressing M1-CCD cells, aldosterone stimulates the sustained activation of ERK1/2 up to at least 120 min after hormone treatment through a mechanism that is sensitive to MR antagonism (Fig. 9). Aldosterone-induced ERK activation at 2 min was sensitive to the general PKC antagonist chelerythrine chloride [21]. We found that only the later phase of ERK1/2 activity was affected by PKD1 suppression, suggesting a role for PKC isoforms in the initiation of ERK1/2 activation and a role for PKD1 in contributing to the stabilization of ERK1/2 activity. Further integration of aldosterone-induced signalling occurs at the level of EGFR *trans*-activation. The activation of PKD1 by aldosterone is dependent on EGFR trans-activation [32] as is aldosterone-induced ERK1/2 activation [50]. This is indicative of EGFR activation having roles in both initiating and sustaining the aldosterone-induced ERK1/2 activity. The activation of ERK1/2 in response to estradiol is dependent on PKCδ activation [51] and ERK activation in response to growth factors and cytokines are PKCδ-dependent [52-54].

PKD1 over-expression potentiates GPCR agonist-induced cell growth and ERK1/2 activation in Swiss-3T3 fibroblasts [17]. In wild type Swiss 3T3 cells vasopressin stimulated ERK1/2 activation for up to 15 min, however heterologous over-expression of PKD1 resulted in increased activation at early time points (5 to 15 min) that was sustained for at least 240 min. This effect of PKD1 coincided temporally with its activation and autophosphorylation. In this present study we found that aldosterone-induced ERK1/2 activity also

coincided with PKD1 activation and in common with the vasopressin work, the earliest phase in aldosterone-induced ERK1/2 activation was not PKD1-dependent. Vasopressin did not stimulate proliferation of Swiss 3T3 fibroblasts unless PKD1 was over expressed and bombesin-induced proliferation of wild-type 3T3 cells was increased by 2 to 3-fold with PKD1 over-expression. We found that aldosterone-induced CCD cell proliferation was completely blocked with PKD1 suppression confirming the important role of this kinase in both GPCR agonist and aldosterone-induced cell growth.

The mechanism by which PKD1 sustains ERK1/2 activity is unclear. PKD1 is a key regulator of sub-cellular trafficking events and since ERK1/2 re-distributed to different sites in the cells following aldosterone treatment, it is possible that PKD1 not only facilitates rapid nuclear localization of ERK1/2 but also modulates subsequent redistribution when the two kinases are not physically associated. Additional work is required to fully establish the mechanism of sustaining ERK1/2 activation. Since PKD1 also facilitates the nuclear localization of MR, the transcriptional responses initiated by aldosterone may also impact on ERK1/2 activity so making the PKD1 effect on sustained ERK1/2 activity an indirect linkage. Similar coupling of PKD1 activation to sustaining ERK1/2 activity was not observed following EGF treatment of Swiss 3T3 fibroblast cells [17]. However the *trans*-activation of EGFR by aldosterone is required for PKD1 and ERK1/2 activation by the hormone [23, 32]. In this present study we also show that aldosterone-induced cell proliferation is sensitive to EGFR antagonism. One explanation is that the activation of EGFR by aldosterone occurs via a different mechanism to the dimerization and *trans*-phosphorylation of EGFR stimulated by EGF [32]. Alternatively, EGFR *trans*-activation is modulated by other, synergistic signalling processes when stimulated by aldosterone. The activation of c-Src tyrosine kinase is one of the earliest signalling responses to aldosterone in renal cells [31] and the phosphorylation of EGFR by c-Src at Tyr 845 is essential for aldosterone induced PKD1 activation [32]. The simultaneous activation of c-Src is therefore an important modulator of the downstream effects of EGFR *trans*-activation by aldosterone. The effects of PKD1 activation on cell growth are multi-factorial. Not only does PKD1 stabilize ERK activity but also increases the duration of RSK and FAK activity in response to GPCR agonists [17, 55]. PKD1 also suppresses JNK activity to inhibit apoptosis [56, 57]. PKD1 is therefore a crucial regulator of cell growth.

The role of aldosterone-induced cell growth in the repair of physical damage to the renal epithelium remains unclear, however, aldosterone is essential for the differentiation and proliferation of renal embryonic stem cells in renal tubule formation [45]. The proliferation of the renal epithelium seen in ARPKD is mainly localized to the MR-expressing distal nephron [58]. This is circumstantial evidence for a role for aldosterone in aberrant proliferation in the distal nephron. Silencing of the ARPKD-associated fibrocystin gene in HEK293 cells made them hyper-proliferative to EGF and resulted in over-activation of ERK1/2 in response to EGF treatment [59]. Aberrant expression of Heparin binding EGF is detected in ARPKD and EGF antagonistic antibodies suppress the mitogenicity of cystic fluid [60]. Hypertension has long been associated with the progression of ADPKD [61, 62]. Blockade of the rennin-angiotensin-aldosterone system (RAAS) to suppress hypertension as a means of slowing cyst formation in ADPKD is the subject of current clinical investigation [63]. Antagonism of the aldosterone induced signalling pathways that are coupled to EGFR *trans*-activation leading to cell proliferation may also provide a route for pharmacological intervention in treating ADPKD and ARPKD. This study provides new insights into the molecular mechanisms, in particular the role of novel protein kinase D, for proliferative effects of aldosterone through 'non-genomic' signalling cascades in renal CCD.

ACKNOWLEDGEMENT

This work were supported by programme grant 060809/Z/00 from the Wellcome Trust and by the Higher Education Authority of Ireland under the Programme for Research in Third Level Institutions (PRTLI) Cycles 3 and 4 (to BJH) and by a Science Foundation of Ireland, Research Frontiers Programme grant (to WT).

REFERENCES

[1] R.E. Booth, J.P. Johnson, J.D. Stockand, Aldosterone, Adv Physiol Educ 26(1-4) (2002) 8-20.

[2] M.L. Gumz, M.P. Popp, C.S. Wingo, B.D. Cain, Early transcriptional effects of aldosterone in a mouse inner medullary collecting duct cell line, Am J Physiol Renal Physiol 285(4) (2003) F664-673.

[3] M. Robert-Nicoud, M. Flahaut, J.M. Elalouf, M. Nicod, M. Salinas, M. Bens, A. Doucet, P. Wincker, F. Artiguenave, J.D. Horisberger, A. Vandewalle, B.C. Rossier, D. Firsov, Transcriptome of a mouse kidney cortical collecting duct cell line: effects of aldosterone and vasopressin, Proc Natl Acad Sci U S A 98(5) (2001) 2712-2716.

[4] H. Garty, L.G. Palmer, Epithelial sodium channels: function, structure, and regulation, Physiol Rev 77(2) (1997) 359-396.

[5] F. Verrey, Transcriptional control of sodium transport in tight epithelial by adrenal steroids, J Membr Biol 144(2) (1995) 93-110.

[6] P.M. Snyder, D.R. Olson, B.C. Thomas, Serum and glucocorticoid-regulated kinase modulates Nedd4-2-mediated inhibition of the epithelial Na+ channel, J Biol Chem 277(1) (2002) 5-8.

[7] A. Nishiyama, L. Yao, Y. Fan, M. Kyaw, N. Kataoka, K. Hashimoto, Y. Nagai, E. Nakamura, M. Yoshizumi, T. Shokoji, S. Kimura, H. Kiyomoto, K. Tsujioka, M. Kohno, T. Tamaki, F. Kajiya, Y. Abe, Involvement of aldosterone and mineralocorticoid receptors in rat mesangial cell proliferation and deformability, Hypertension 45(4) (2005) 710-716.

[8] Y. Terada, T. Kobayashi, H. Kuwana, H. Tanaka, S. Inoshita, M. Kuwahara, S. Sasaki, Aldosterone stimulates proliferation of mesangial cells by activating mitogen-activated protein kinase 1/2, cyclin D1, and cyclin A, J Am Soc Nephrol 16(8) (2005) 2296-2305.

[9] F. Xiao, J.R. Puddefoot, G.P. Vinson, Aldosterone mediates angiotensin II-stimulated rat vascular smooth muscle cell proliferation, J Endocrinol 165(2) (2000) 533-536.

[10] S.E. Campbell, J.S. Janicki, K.T. Weber, Temporal differences in fibroblast proliferation and phenotype expression in response to chronic administration of angiotensin II or aldosterone, J Mol Cell Cardiol 27(8) (1995) 1545-1560.

[11] J.W. Funder, The nongenomic actions of aldosterone, Endocr Rev 26(3) (2005) 313-321.

[12] W. Thomas, V. McEneaney, B.J. Harvey, Rapid responses to steroid hormones in the kidney, Nephron Physiol 107(1) (2007) p1-9.

[13] V. Urbach, E. Van Kerkhove, D. Maguire, B.J. Harvey, Rapid activation of KATP channels by aldosterone in principal cells of frog skin, J Physiol 491 (Pt 1) (1996) 111-120.

[14] M. Gekle, R. Freudinger, S. Mildenberger, S. Silbernagl, Aldosterone interaction with epidermal growth factor receptor signaling in MDCK cells, Am J Physiol Renal Physiol 282(4) (2002) F669-679.

[15] M. Gekle, N. Golenhofen, H. Oberleithner, S. Silbernagl, Rapid activation of Na+/H+ exchange by aldosterone in renal epithelial cells requires Ca2+ and stimulation of a plasma membrane proton conductance, Proc Natl Acad Sci U S A 93(19) (1996) 10500-10504.

[16] M. Gekle, R. Freudinger, S. Mildenberger, K. Schenk, I. Marschitz, H. Schramek, Rapid activation of Na+/H+-exchange in MDCK cells by aldosterone involves MAP-kinase ERK1/2, Pflugers Arch 441(6) (2001) 781-786.

[17] J. Sinnett-Smith, E. Zhukova, N. Hsieh, X. Jiang, E. Rozengurt, Protein kinase D potentiates DNA synthesis induced by Gq-coupled receptors by increasing the duration of ERK signaling in swiss 3T3 cells, J Biol Chem 279(16) (2004) 16883-16893.

[18] S. Michlig, A. Mercier, A. Doucet, L. Schild, J.D. Horisberger, B.C. Rossier, D. Firsov, ERK1/2 controls Na,K-ATPase activity and transepithelial sodium transport in the principal cell of the cortical collecting duct of the mouse kidney, J Biol Chem 279(49) (2004) 51002-51012.

[19] E. Rozengurt, O. Rey, R.T. Waldron, Protein kinase D signaling, J Biol Chem 280(14) (2005) 13205-13208.

[20] N. Tsybouleva, L. Zhang, S. Chen, R. Patel, S. Lutucuta, S. Nemoto, G. DeFreitas, M. Entman, B.A. Carabello, R. Roberts, A.J. Marian, Aldosterone, through novel signaling proteins, is a fundamental molecular bridge between the genetic defect and the cardiac phenotype of hypertrophic cardiomyopathy, Circulation 109(10) (2004) 1284-1291.

[21] F. Markos, V. Healy, B.J. Harvey, Aldosterone rapidly activates Na+/H+ exchange in M-1 cortical collecting duct cells via a PKC-MAPK pathway, Nephron Physiol 99(1) (2005) p1-9.

[22] K. Rossol-Haseroth, Q. Zhou, S. Braun, B. Boldyreff, E. Falkenstein, M. Wehling, R.M. Losel, Mineralocorticoid receptor antagonists do not block rapid ERK activation by aldosterone, Biochem Biophys Res Commun 318(1) (2004) 281-288.

[23] M. Gekle, R. Freudinger, S. Mildenberger, S. Silbernagl, Rapid actions of aldosterone on cells from renal epithelium: the possible role of EGF-receptor signaling, Steroids 67(6) (2002) 499-504.

[24] J.R. Gregoire, V.E. Torres, K.E. Holley, G.M. Farrow, Renal epithelial hyperplastic and neoplastic proliferation in autosomal dominant polycystic kidney disease, Am J Kidney Dis 9(1) (1987) 27-38.

[25] L. Al-Bhalal, M. Akhtar, Molecular basis of autosomal recessive polycystic kidney disease (ARPKD), Adv Anat Pathol 15(1) (2008) 54-58.

[26] C.J. Ward, H. Turley, A.C. Ong, M. Comley, S. Biddolph, R. Chetty, P.J. Ratcliffe, K. Gattner, P.C. Harris, Polycystin, the polycystic kidney disease 1 protein, is expressed by epithelial cells in fetal, adult, and polycystic kidney, Proc Natl Acad Sci U S A 93(4) (1996) 1524-1528.

[27] J.G. Yates-Bell, Hypertension in polycystic disease, Br J Urol 29(4) (1957) 371-373.

[28] A. Masoumi, B. Reed-Gitomer, C. Kelleher, R.W. Schrier, Potential pharmacological interventions in polycystic kidney disease, Drugs 67(17) (2007) 2495-2510.

[29] C. Korbmacher, A.S. Segal, G. Fejes-Toth, G. Giebisch, E.L. Boulpaep, Whole-cell currents in single and confluent M-1 mouse cortical collecting duct cells, J Gen Physiol 102(4) (1993) 761-793.

[30] V. McEneaney, B.J. Harvey, W. Thomas, Aldosterone Regulates Rapid Trafficking of Epithelial Sodium Channel Subunits in Renal Cortical Collecting Duct Cells via Protein Kinase D Activation, Mol Endocrinol 22(4) (2008) 881-892.

[31] S. Braun, R. Losel, M. Wehling, B. Boldyreff, Aldosterone rapidly activates Src kinase in M-1 cells involving the mineralocorticoid receptor and HSP84, FEBS Lett 570(1-3) (2004) 69-72.

[32] V. McEneaney, B.J. Harvey, W. Thomas, Aldosterone rapidly activates protein kinase D via a mineralocorticoid receptor/EGFR trans-activation pathway in the M1 kidney CCD cell line, J Steroid Biochem Mol Biol 107(3-5) (2007) 180-190.

[33] B.A. Stoos, A. Naray-Fejes-Toth, O.A. Carretero, S. Ito, G. Fejes-Toth, Characterization of a mouse cortical collecting duct cell line, Kidney Int 39(6) (1991) 1168-1175.

[34] S. Kato, H. Endoh, Y. Masuhiro, T. Kitamoto, S. Uchiyama, H. Sasaki, S. Masushige, Y. Gotoh, E. Nishida, H. Kawashima, D. Metzger, P. Chambon, Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase, Science 270(5241) (1995) 1491-1494.

[35] C. Le Moellic, A. Ouvrard-Pascaud, C. Capurro, F. Cluzeaud, M. Fay, F. Jaisser, N. Farman, M. Blot-Chabaud, Early nongenomic events in aldosterone action in renal collecting duct cells: PKCalpha activation, mineralocorticoid receptor phosphorylation, and cross-talk with the genomic response, J Am Soc Nephrol 15(5) (2004) 1145-1160.

[36] R. Verani, P. Walker, F.G. Silva, Renal cystic disease of infancy: results of histochemical studies. A report of the Southwest Pediatric Nephrology Study Group, Pediatr Nephrol 3(1) (1989) 37-42.

[37] S. Horikoshi, S. Kubota, G.R. Martin, Y. Yamada, P.E. Klotman, Epidermal growth factor (EGF) expression in the congenital polycystic mouse kidney, Kidney Int 39(1) (1991) 57-62.

[38] R.H. Chen, C. Sarnecki, J. Blenis, Nuclear localization and regulation of erk- and rsk-encoded protein kinases, Mol Cell Biol 12(3) (1992) 915-927.

[39] A.G. Turjanski, J.P. Vaque, J.S. Gutkind, MAP kinases and the control of nuclear events, Oncogene 26(22) (2007) 3240-3253.

[40] P. Lenormand, C. Sardet, G. Pages, G. L'Allemain, A. Brunet, J. Pouyssegur, Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts, J Cell Biol 122(5) (1993) 1079-1088.

[41] M. Adachi, M. Fukuda, E. Nishida, Nuclear export of MAP kinase (ERK) involves a MAP kinase kinase (MEK)-dependent active transport mechanism, J Cell Biol 148(5) (2000) 849-856.

[42] R. Rocha, C.T. Stier, Jr., I. Kifor, M.R. Ochoa-Maya, H.G. Rennke, G.H. Williams, G.K. Adler, Aldosterone: a mediator of myocardial necrosis and renal arteriopathy, Endocrinology 141(10) (2000) 3871-3878.

[43] A. Chrysostomou, G. Becker, Spironolactone in addition to ACE inhibition to reduce proteinuria in patients with chronic renal disease, N Engl J Med 345(12) (2001) 925-926.

[44] A. Nishiyama, L. Yao, Y. Nagai, K. Miyata, M. Yoshizumi, S. Kagami, S. Kondo, H. Kiyomoto, T. Shokoji, S. Kimura, M. Kohno, Y. Abe, Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats, Hypertension 43(4) (2004) 841-848.

[45] W.W. Minuth, L. Denk, K. Hu, The role of polyester interstitium and aldosterone during structural development of renal tubules in serum-free medium, Biomaterials 28(30) (2007) 4418-4428.

[46] G. L'Allemain, S. Paris, J. Pouyssegur, Growth factor action and intracellular pH regulation in fibroblasts. Evidence for a major role of the Na+/H+ antiport, J Biol Chem 259(9) (1984) 5809-5815.

[47] A. Pandiella, L. Beguinot, T.J. Velu, J. Meldolesi, Transmembrane signalling at epidermal growth factor receptors overexpressed in NIH 3T3 cells. Phosphoinositide hydrolysis, cytosolic Ca2+ increase and alkalinization correlate with epidermal-growth-factor-induced cell proliferation, Biochem J 254(1) (1988) 223-228.

[48] E. Hendron, J.D. Stockand, Activation of mitogen-activated protein kinase (mitogen-activated protein kinase/extracellular signal-regulated kinase) cascade by aldosterone, Mol Biol Cell 13(9) (2002) 3042-3054.

[49] S.Y. Chen, J. Wang, W. Liu, D. Pearce, Aldosterone responsiveness of A6 cells is restored by cloned rat mineralocorticoid receptor, Am J Physiol 274(1 Pt 1) (1998) C39-46.

[50] C. Grossmann, A. Benesic, A.W. Krug, R. Freudinger, S. Mildenberger, B. Gassner, M. Gekle, Human mineralocorticoid receptor expression renders cells responsive for nongenotropic aldosterone actions, Mol Endocrinol 19(7) (2005) 1697-1710.

[51] V.G. Keshamouni, R.R. Mattingly, K.B. Reddy, Mechanism of 17-beta-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-delta, J Biol Chem 277(25) (2002) 22558-22565.

[52] R. Ginnan, B.J. Guikema, H.A. Singer, D. Jourd'heuil, PKC-delta mediates activation of ERK1/2 and induction of iNOS by IL-1beta in vascular smooth muscle cells, Am J Physiol Cell Physiol 290(6) (2006) C1583-1591.

[53] R. Ginnan, P.J. Pfleiderer, K. Pumiglia, H.A. Singer, PKC-delta and CaMKII-delta 2 mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle, Am J Physiol Cell Physiol 286(6) (2004) C1281-1289.

[54] R. Ginnan, H.A. Singer, PKC-delta-dependent pathways contribute to PDGF-stimulated ERK1/2 activation in vascular smooth muscle, Am J Physiol Cell Physiol 288(6) (2005) C1193-1201.

[55] J. Sinnett-Smith, E. Zhukova, O. Rey, E. Rozengurt, Protein kinase D2 potentiates MEK/ERK/RSK signaling, c-Fos accumulation and DNA synthesis induced by bombesin in Swiss 3T3 cells, J Cell Physiol 211(3) (2007) 781-790.

[56] I. Brandlin, T. Eiseler, R. Salowsky, F.J. Johannes, Protein kinase C(mu) regulation of the JNK pathway is triggered via phosphoinositide-dependent kinase 1 and protein kinase C(epsilon), J Biol Chem 277(47) (2002) 45451-45457.

[57] I. Brandlin, S. Hubner, T. Eiseler, M. Martinez-Moya, A. Horschinek, A. Hausser, G. Link, S. Rupp, P. Storz, K. Pfizenmaier, F.J. Johannes, Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways, J Biol Chem 277(8) (2002) 6490-6496.

[58] T. Nadasdy, Z. Laszik, G. Lajoie, K.E. Blick, D.E. Wheeler, F.G. Silva, Proliferative activity of cyst epithelium in human renal cystic diseases, J Am Soc Nephrol 5(7) (1995) 1462-1468.

[59] J. Yang, S. Zhang, Q. Zhou, H. Guo, K. Zhang, R. Zheng, C. Xiao, PKHD1 gene silencing may cause cell abnormal proliferation through modulation of intracellular calcium in autosomal recessive polycystic kidney disease, J Biochem Mol Biol 40(4) (2007) 467-474.

[60] K. MacRae Dell, R. Nemo, W.E. Sweeney, Jr., E.D. Avner, EGF-related growth factors in the pathogenesis of murine ARPKD, Kidney Int 65(6) (2004) 2018-2029.

[61] A.P. Shapiro, F.H. Leenen, S.J. Galla, D.P. Redmond, A.H. Vagnucci, R.H. McDonald, Jr., Blood pressure regulation during chronic renal failure due to polycystic renal disease, Acta Physiol Lat Am 24(5) (1974) 432-438.

[62] H. Danielsen, E.B. Pedersen, A.H. Nielsen, P. Herlevsen, H.J. Kornerup, V. Posborg, Expansion of extracellular volume in early polycystic kidney disease, Acta Med Scand 219(4) (1986) 399-405.

[63] A.B. Chapman, Approaches to testing new treatments in autosomal dominant polycystic kidney disease: insights from the CRISP and HALT-PKD studies, Clin J Am Soc Nephrol 3(4) (2008) 1197-1204.

[64] A. Naray-Fejes-Toth, C. Canessa, E.S. Cleaveland, G. Aldrich, G. Fejes-Toth, sgk is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial na+ channels, J Biol Chem 274(24) (1999) 16973-16978.

[65] A. Naray-Fejes-Toth, G. Fejes-Toth, The sgk, an aldosterone-induced gene in mineralocorticoid t arget cells, regulates the epithelial sodium channel, Kidney Int 57(4) (2000) 1290-1294.

FIGURE LEGENDS

Fig. 1. **Aldosterone promotes the growth of M1-CCD cells.** Aldosterone stimulated cell growth was measured using the MTT cell proliferation assay. The assay was performed on wild-type M1-CCD and PKD1 suppressed M1-CCD cells (A). Cells were left untreated (0) or treated with aldosterone (Aldo) or vehicle control (Con) for 48 h. The MTT assay was performed and absorbance measurements at 570 nm were recorded. Values represent the means of four separate experiments ***, $p < 0.001$ versus wild-type M1-CCD cells treated with aldosterone. M1-CCD cells were pre-treated with the PKCδ antagonist rottlerin (20 μM) for 30 min and the appropriate inhibitor vehicle prior to addition of aldosterone or vehicle control for 48 h (B). The MTT cell growth assay was performed on M1-CCD WT cells were pre-treated with the ERK1/2 antagonist PD98059 (1 μM) for 30 min and the appropriate inhibitor vehicle prior to addition of aldosterone or vehicle control for 48h (C). The incidence of apoptosis, as measured by cytoplasmic nucleosome enrichment in the M1-CCD wild-type or PKD1 suppressed M1-CCD cells treated with 10 nM aldosterone for 48 h was determined (D). The background A_{405} value was subtracted from the absorbance values for each experimental condition. In order to calculate the enrichment factor, the background corrected absorbance value for aldosterone-treated cells was divided by that for vehicle-treated cells and the results plotted in a bar chart, mean ± SEM. The assay was carried out in duplicate for each condition and each experiment repeated twice.

Fig. 2. **PKD1 modulates aldosterone-induced ERK1/2 activation.** Wild-type M1-CCD cells (A) and cells suppressed in PKD1 expression (B) were treated with aldosterone (+) for 2, 5, 10, 20, 30, 60, 90, and 120 min or treated with vehicle control (-) for the same time periods. Western blotting was performed using a phospho-ERK1/2 Thr202/Tyr204 specific antibody. Membranes were then stripped and re-probed with a total ERK1/2. Densitometry was performed on the 44 kDa ERK1 and the 42 kDa ERK2 bands separately. Values represent the means of three separate experiments ***, $p < 0.001$ versus control.

Fig. 3. **Aldosterone promotes the association of PKD1 with ERK1/2 MAPK.** Wild-type M1-CCD cells were treated with aldosterone (+) for 2 min or treated with vehicle control (-) for the same time periods. Cells were also pre-treated with the PKCδ antagonist rottlerin (20μM) for 30 min before aldosterone addition. Western blotting was performed using a phospho-ERK1/2 Thr202/Tyr204-specific antibody. Membranes were then stripped and re-probed with a total ERK1/2 antibody (A). The interaction between PKD1 and ERK1/2 MAP kinase was investigated by co-immunoprecipitation (B). Lysates were prepared from M1-CCD cells either treated with 10 nM aldosterone (+) or vehicle control (-) for 2 min or left untreated (0). PKD1 was immunoprecipitated from the lysates using a total PKD1 antibody coupled to protein G sepharose. Immunoprecipitated proteins were separated by SDS-PAGE. Western blotting was performed using a total ERK1/2 antibody or a total PKD1 antibody.

Fig. 4. **Receptor involvement in aldosterone-induced proliferation.** M1-CCD cells were pre-treated with the MR antagonist spironolactone (10 μM) for 30 min (A) or with the EGFR antagonist Tyrphostin AG1478 (1 μM) for 30 min (B) prior to addition of aldosterone (Aldo) or vehicle control (Con) for 48 h. The MTT proliferation assay was performed and absorbance values at 570 nm were recorded and subjected to statistical analysis. Values represent the means of four separate experiments ***, $p < 0.001$ versus control.

Fig. 5. **MR involvement in aldosterone-induced ERK1/2 activation.** M1-CCD cells were left untreated (0) or treated with aldosterone (10 nM) or vehicle control for 2 min alone or in the presence of the MR inhibitor RU28318 (10 μM) for 30 min or followed by aldosterone or vehicle control for 2 min or 120 min.

Lysates were separated by SDS-PAGE and Western blotted using a phospho-ERK1/2 Thr202/Tyr204-specific antibody. The membranes were then stripped and re-probed with a total ERK1/2 antibody. Values represent the means of three separate experiments ***, $p < 0.001$ versus aldosterone treated M1-CCD cells.

Fig. 6. **Aldosterone induces nuclear translocation of ERK1/2.** Wild-type M1-CCD (A) and PKD1 suppressed M1-CCD cells (B) were grown on 8-well chamber slides. Cells were left untreated or treated with aldosterone for 2 min. Cells were fixed and the sub-cellular distribution of ERK1/2 was determined by immunofluorescence using an ERK1/2 specific monoclonal antibody and detected using a rabbit anti-mouse Alexa 488 conjugate (green), nuclei were stained with DAPI (red). Imaging was performed using a Zeiss LSM 510 meta confocal microscope at x63 magnification. Representative single XY focal plain images through the mid region of the cell monolayer are shown with separate 488 nm and 364 nm channels and a merged image of both channels. Bar charts depict the mean nuclear fluorescence intensity after ERK1/2 immunocytochemistry in M1-CCD and PKD1 suppressed M1-CCD cells, before and after aldosterone treatment for 2 min. Statistical analysis was performed using a student's t test, *** $p < 0.0001$. (WT control n=37, WT Aldo. n=39, PKD1 KD control n=39, PKD1 KD Aldo. n=40). AU: arbitrary units.

Fig. 7. **ERK1/2 localization after aldosterone treatment for 30 min.** Wild-type M1-CCD and PKD1 suppressed M1-CCD cells were grown on 8-well chamber slides. Cells treated with aldosterone for 30 min, fixed and the sub-cellular distribution of ERK1/2 was determined by immunofluorescence using an ERK1/2 specific monoclonal antibody and detected using a rabbit anti-mouse Alexa 488 conjugate (green), nuclei were stained with DAPI (red). Imaging was performed using a Zeiss LSM 510 meta confocal microscope at x63 magnification. Representative single XY focal plain images through the mid region of the cell monolayer are shown with separate 488 nm and 364 nm channels and a merged image of both channels.

Fig. 8. **ERK1/2 localization after aldosterone treatment for 120 min.** Wild-type M1-CCD and PKD1 suppressed M1-CCD cells were grown on 8-well chamber slides. Cells treated with aldosterone for 120 min, fixed and the sub-cellular distribution of ERK1/2 was determined by immunofluorescence using an ERK1/2 specific monoclonal antibody and detected using a rabbit anti-mouse Alexa 488 conjugate (green), nuclei were stained with DAPI (red). Imaging was performed using a Zeiss LSM 510 meta confocal microscope at x63 magnification. Representative single XY focal plain images through the mid region of the cell monolayer are shown with separate 488 nm and 364 nm channels and a merged image of both channels.

Fig.9. **Aldosterone-induced ERK activation.** Aldosterone binds to MR to stabilize the interaction of the receptor with specific sequences in target promoters and instigate the assembly of the transcription pre-initiation complex. The *trans*-activation of EGFR by aldosterone is dependent on EGFR phosphorylation by c-Src [32] and leads to activation of a nPKC signalling cascade that results in PKD1 activation [30], and also to the activation of the ERK1/2 cascade [50]. The phosphorylation of MR by ERK1/2 promotes its nuclear localization and transcriptional activity. Concurrent PKD1 activation stabilizes the activation of ERK1/2 and facilitates the translocation of ERK1/2 to the nucleus within 2 min of its activation. ERK1/2 inhabits the nucleus transiently where it phosphorylates transcription factors involved in aldosterone induced gene expression. Changes in the abundance of aldosterone-regulated mRNA species become detectable within 20 min of hormone treatment [64, 65]. Within 30 min ERK1/2 starts to be exported from the nucleus and becomes localized to discrete sites within the cytoplasm. ERK1/2 accumulation at these sites was not observed in cells suppressed in PKD1 expression. After 2 h ERK1/2 is observed at the discrete cytoplamic sites or shows a more general cytoplasmic localization, with very low nuclear abundance.

Figure 1

Click here to download high resolution image

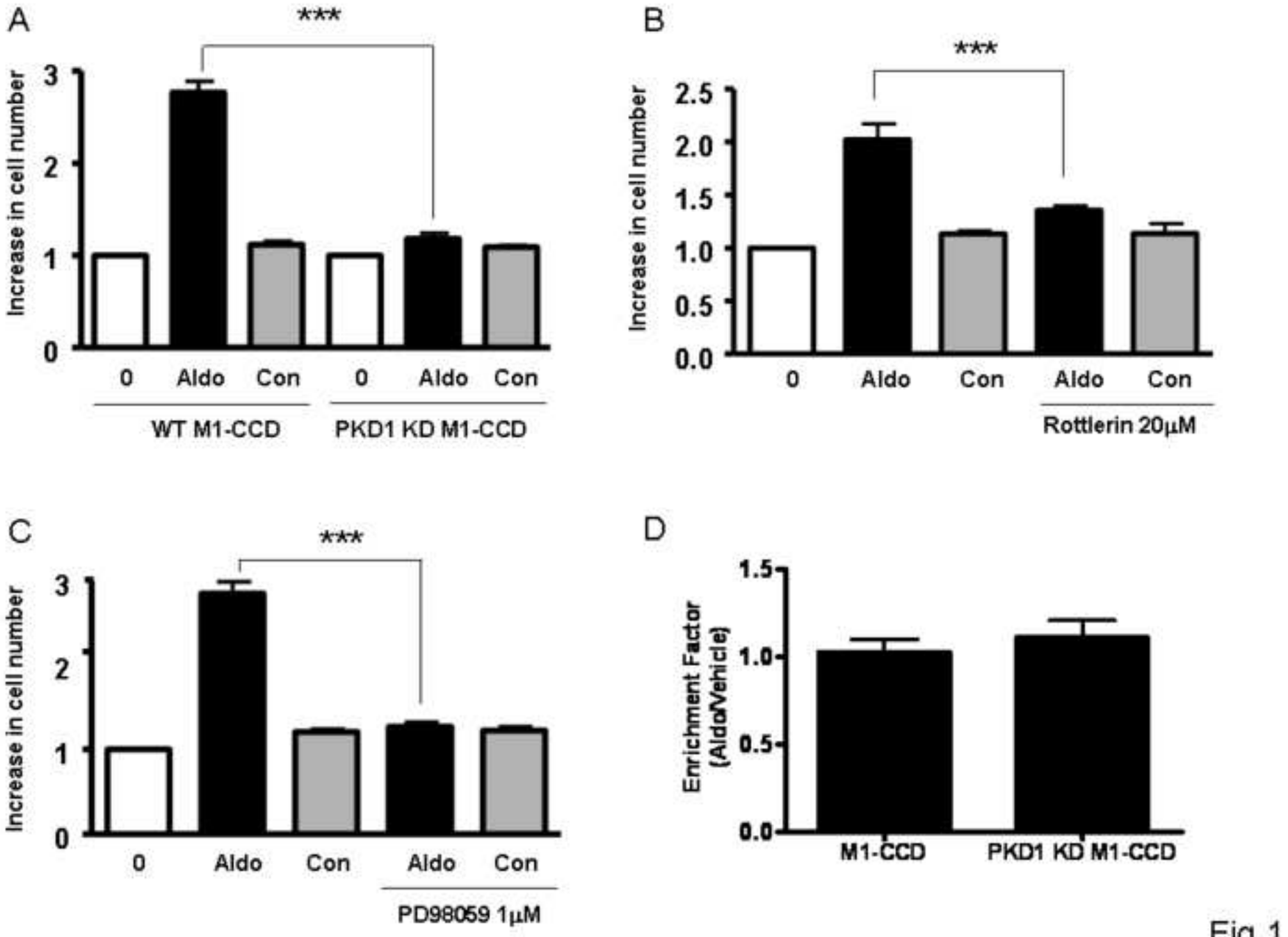

Fig. 1

Figure 2

Click here to download high resolution image

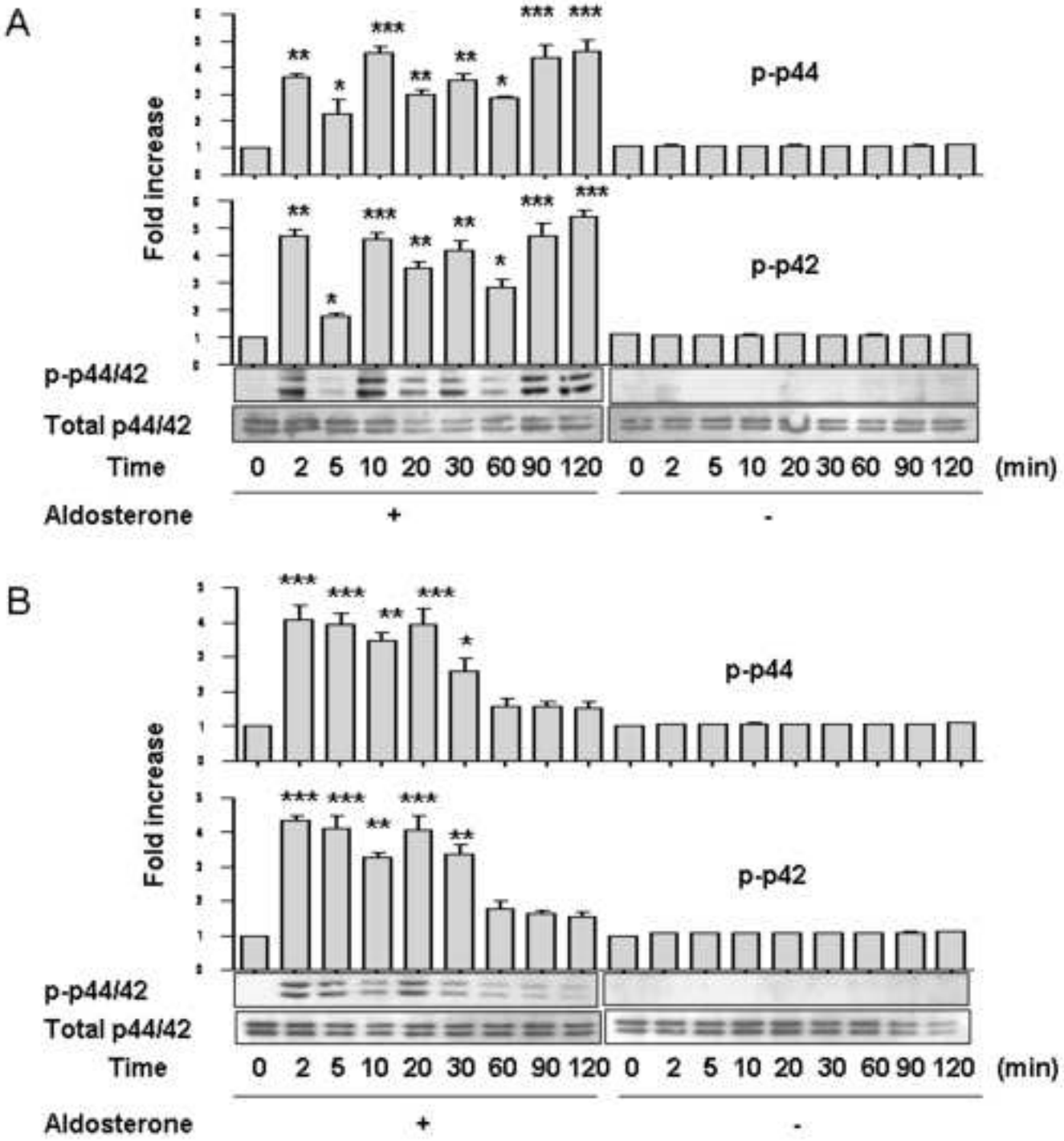

Fig. 2

Figure 3

Click here to download high resolution image

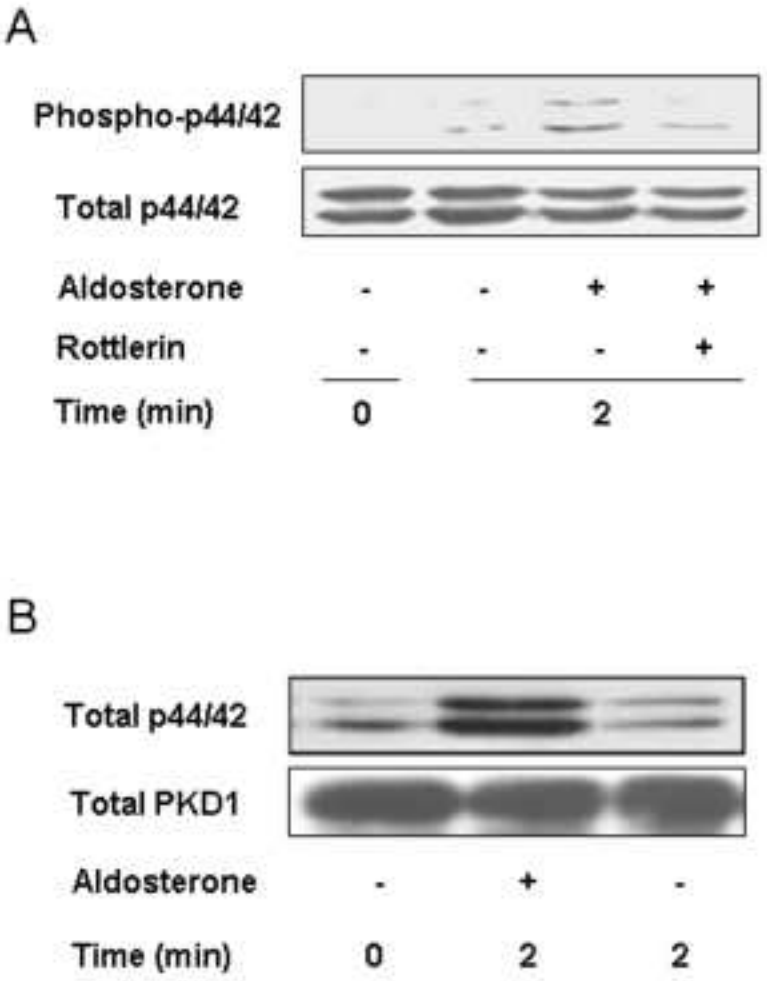

Fig. 3

Figure 4

Click here to download high resolution image

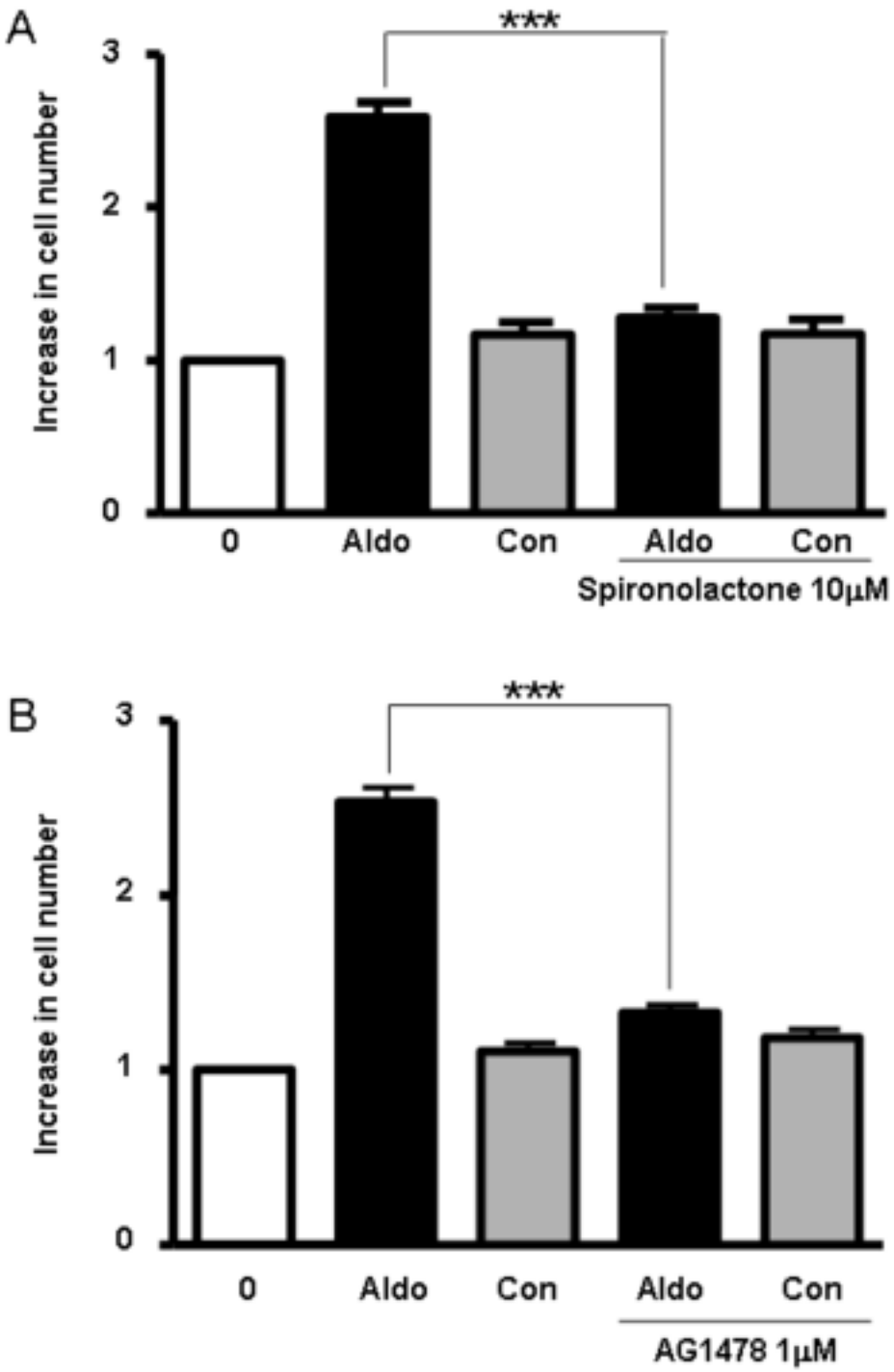

Fig 4

Figure 5

Click here to download high resolution image

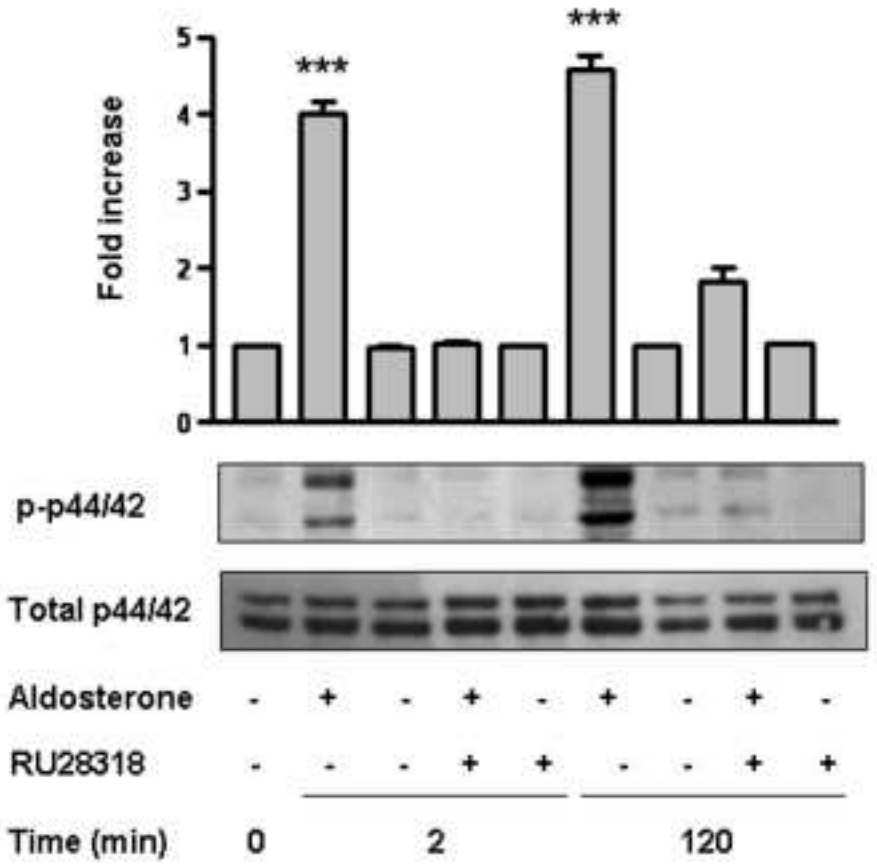

Fig 5

Figure 6

Click here to download high resolution image

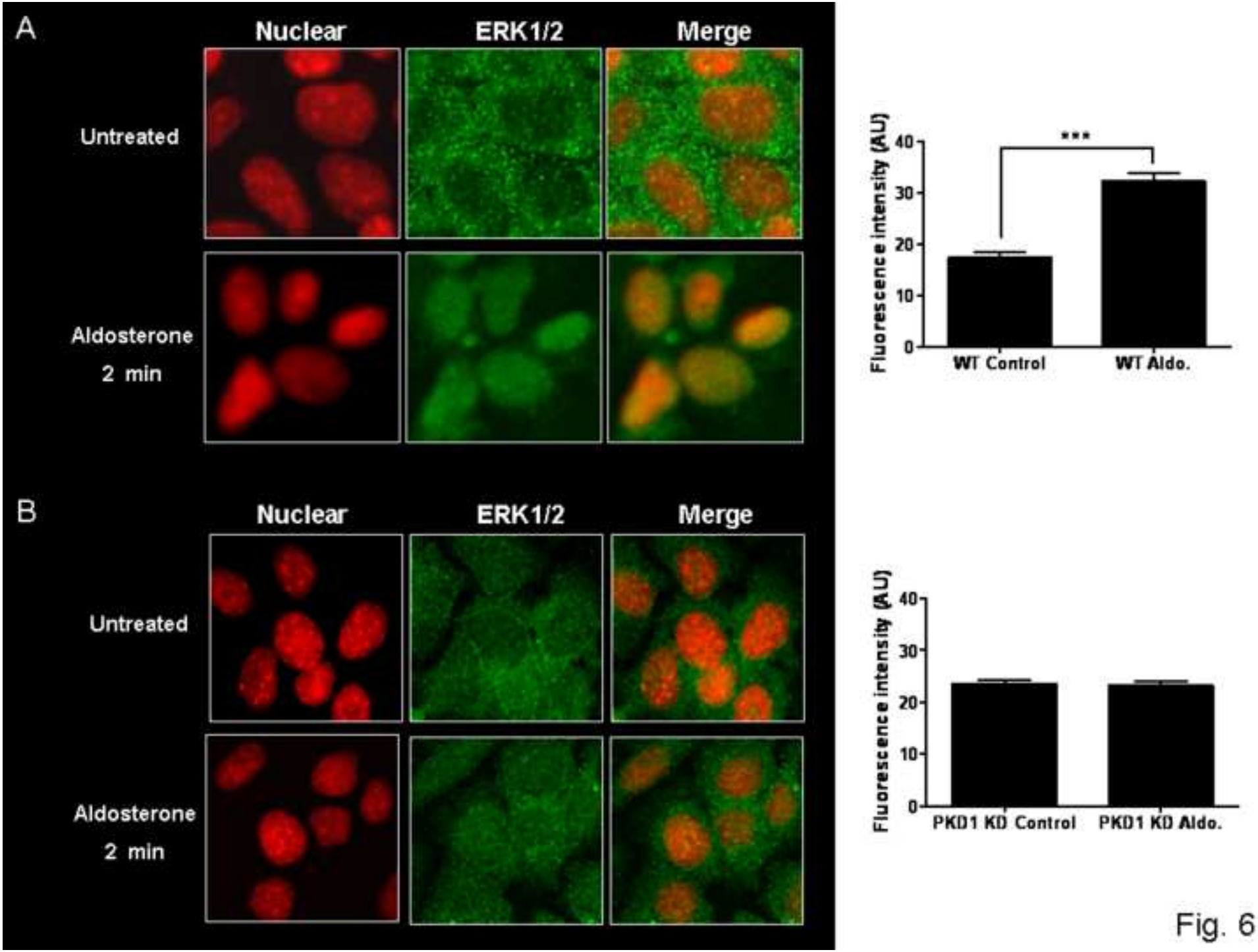

Fig. 6

Figure 7

Click here to download high resolution image

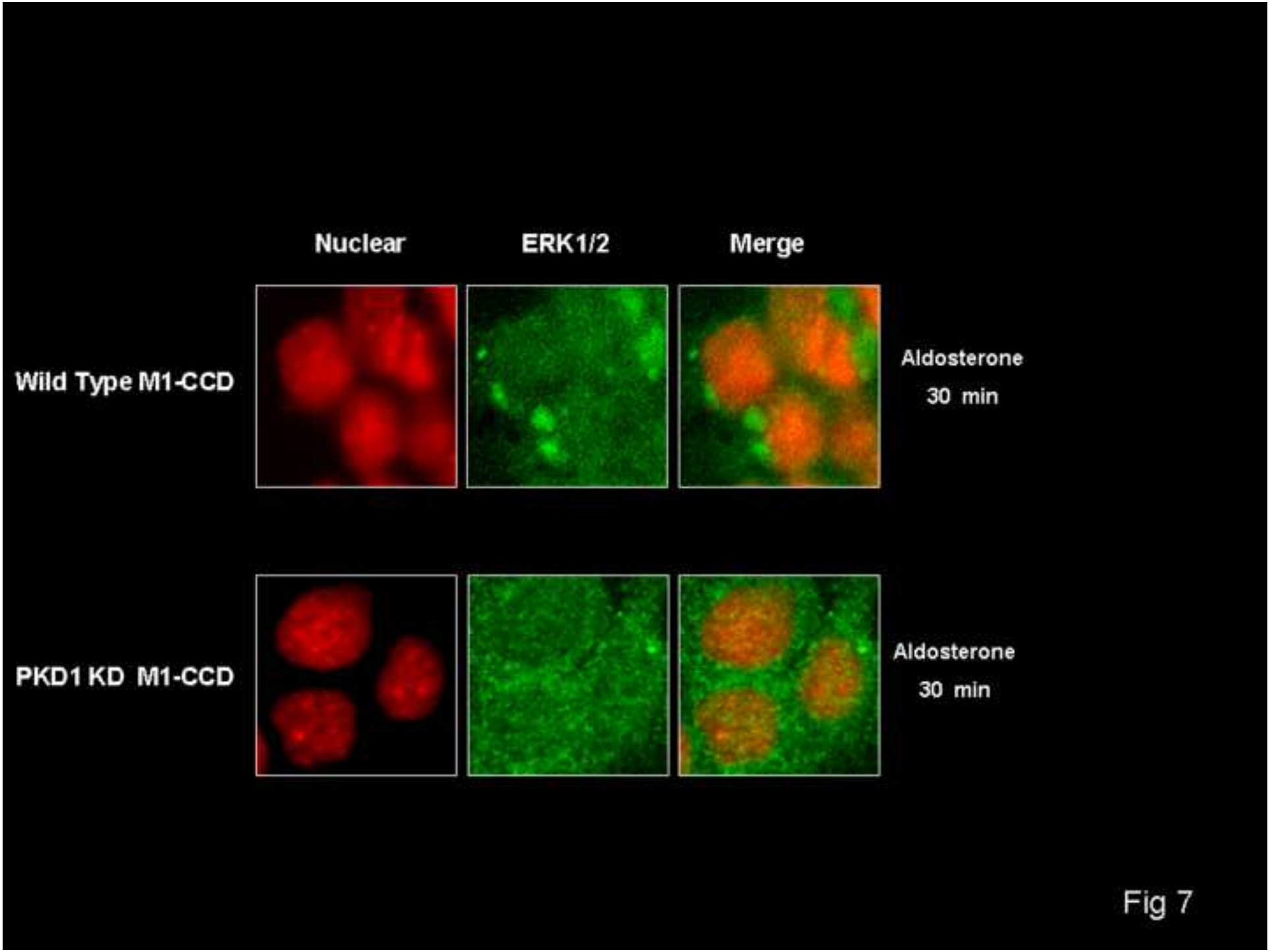

Figure 8

Click here to download high resolution image

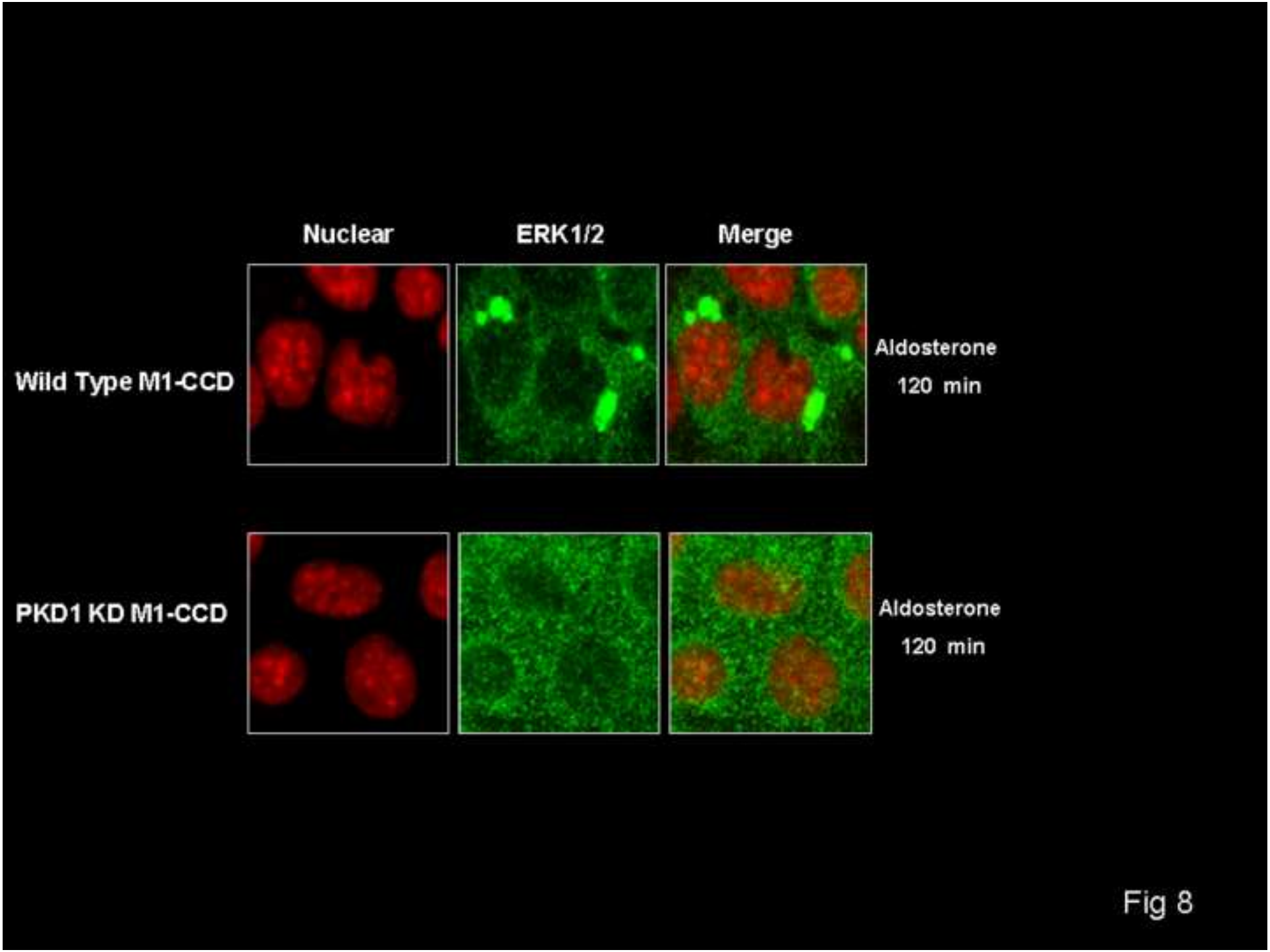

Figure 9

Click here to download high resolution image

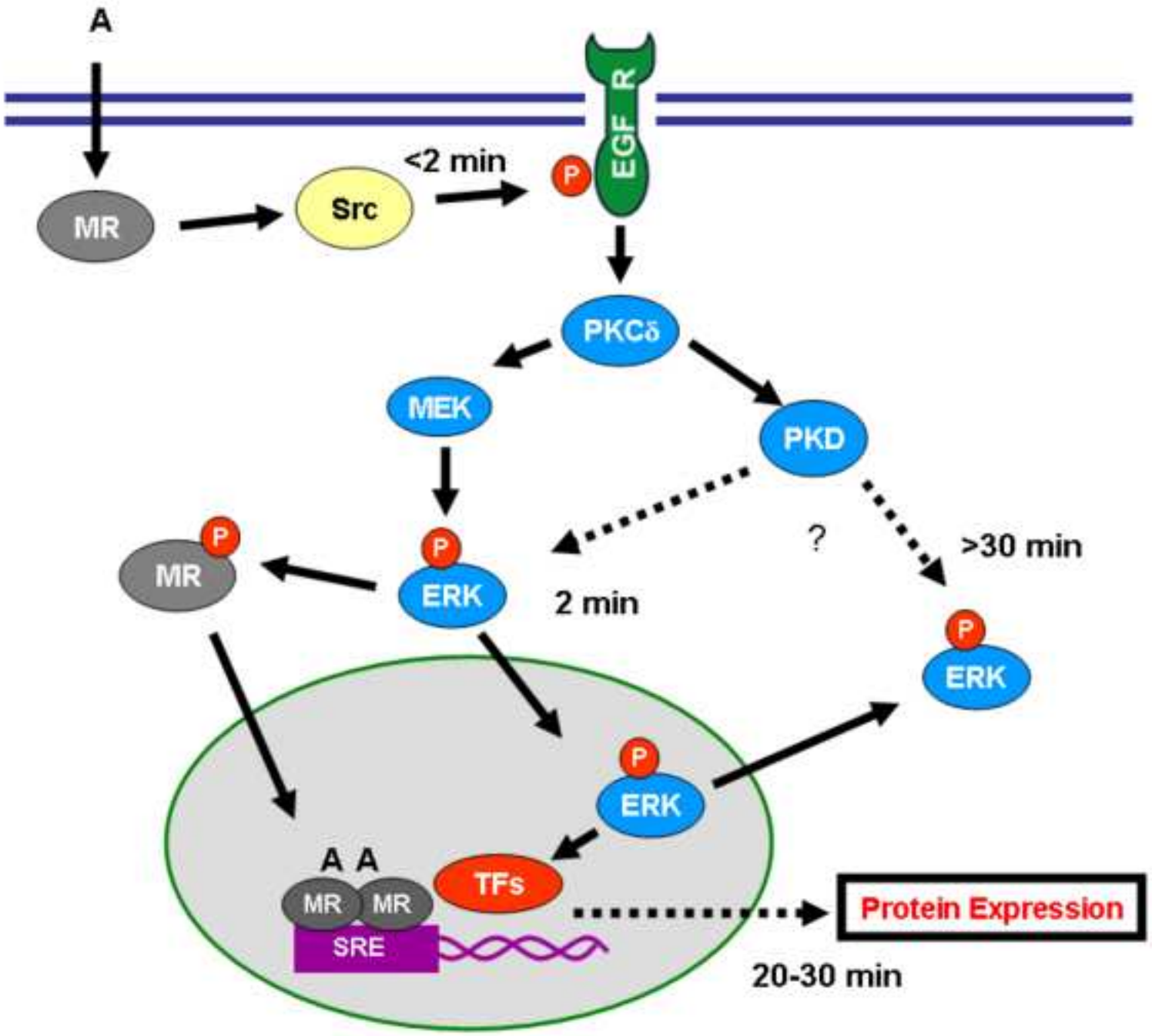

Fig. 10