Trichostatin A Blocks Aldosterone-Induced Na+ Transport And Control Of Serum- And Glucocorticoid-Inducible Kinase 1 In Cortical Collecting Duct Cells

TRICHOSTATIN A BLOCKS ALDOSTERONE-INDUCED Na^+ TRANSPORT AND CONTROL OF SERUM- AND GLUCOCORTICOID-INDUCIBLE KINASE 1 IN CORTICAL COLLECTING DUCT CELLS

Running title: KDAC inhibition and ENaC activity

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Abstract

Background and purpose. Aldosterone stimulates epithelial Na^+ channel (ENaC)-dependent Na^+ retention in the cortical collecting duct (CCD) of the kidney by activating mineralocorticoid receptors (MR) that promote expression of serum and glucocorticoid-inducible kinase 1 (SGK1). This response is critical to blood pressure homeostasis. It has previously been suggested that inhibiting lysine deacetylases (KDACs) can post-transcriptionally disrupt this response by promoting acetylation of the MR. The present study critically evaluates this hypothesis.

Experimental approach. Electrometric and molecular methods were used to define the effects of a pan-KDAC inhibitor (Trichostatin A; TSA) upon the responses to a physiologically relevant concentration of aldosterone (3 nM) in murine $mCCD_{cl1}$ cells.

Key results. Aldosterone augmented ENaC-induced Na^+ absorption and increased SGK1 activity and abundance, as expected. In the presence of TSA these responses were suppressed. TSA-induced inhibition of KDAC was confirmed by increased acetylation of histone H3, H4 and α-tubulin. TSA did not block the electrometric response to insulin, a hormone that activates SGK1 independently of increased transcription, indicating that TSA has no direct effect upon the SGK1 / ENaC pathway.

Conclusions and implications. Inhibition of lysine acetylation suppresses aldosterone-dependent control over the SGK1-ENaC pathway, but does not perturb post-transcriptional signalling, providing a physiological basis for the anti-hypertensive action of KDAC inhibition seen *in vivo*.

Key words: Histone deacetylase, lysine deacetylase, aldosterone, cortical collecting duct, epithelial Na^+ channel (ENaC), sodium transport.

Abbreviations

CCD	Cortical collecting duct
DOCA	Deoxycorticosterone acetate
ENaC	Epithelial Na^+ channel
HDAC	Histone deacetylase
HEK	Human embryonic kidney
I_{amil}	Amiloride-sensitive current

I_{eq}	Equivalent short circuit current
KAT	Lysine acetyl transferase
KDAC	Lysine deacetylase
KDACi	Lysine deacetylase inhibitor
MR	Mineralocorticoid receptor
NDRG1	N-myc downstream regulated gene 1
GR	Glucocorticoid receptor
SGK1	Serum- and glucocorticoid-inducible kinase 1
R_t	Transepithelial resistance
TSA	Trichostatin A
V_t	Transepithelial voltage

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Tables, figure and legends: 1 table and 5 figures have been submitted as part of this manuscript.

What is already known?

- Aldosterone stimulates Na^+ retention by activating mineralocorticoid receptors (MR) in the cortical collecting duct (CCD)
- This anti natriuretic response depends upon MR-mediated changes in gene expression

What this study adds

- As anticipated, TSA consistently promoted acetylation of cytoplasmic and nuclear proteins
- Trichostatin A (TSA) supressed the MR-mediated transcription of *sgk1* and blocked aldosterone-induced Na^+ absorption

Clinical significance

- Hypertension can be treated with drugs that disrupt the response to aldosterone
- Agents that promote protein acetylation may provide a novel means of supressing aldosterone-induced Na^+ retention

Introduction

Protein acetylation is a post translational modification, catalysed by lysine acetyl transferases (KATs, also known as histone acetyl transferases, HATs), that convert positively charged amine groups in lysine residues into neutral amides (see review Falkenberg *et al.*, 2014). The subsequent removal of acetyl residues from proteins modified in this way is catalysed by lysine deacetylases (KDACs, also known as histone deacetylases, HDACs) a zinc-dependent enzyme family (Falkenberg *et al.*, 2014). The acetylation status of many cellular proteins is therefore determined by the relative activities of KATs / KDACs and the acetylation of many cytoplasmic proteins has physiological relevance. Acetylation of the glucocorticoid receptor (GR), for example, modifies the hormone-induced transcription of several genes (Kadiyala *et al.*, 2013; Winkler *et al.*, 2012) whilst acetylation of the cytoplasmic receptors for oestrogen, progesterone and testosterone reduces the ability of sex steroids to regulate gene transcription, an effect that is exploited to suppress the growth of hormone-sensitive tumour cells (Falkenberg *et al.*, 2014). Changes to the acetylation status of cytoplasmic receptors therefore provide a means of modifying responses to steroid hormones (Barnes, 2013; Falkenberg *et al.*, 2014).

Mineralocorticoid receptors (MR) in the cortical collecting duct (CCD) allow aldosterone to promote renal Na^+ reabsorption by evoking expression of genes, such as that encoding serum and glucocorticoid-inducible kinase 1 (SGK1) (Lang *et al.*, 2006), that control the abundance of epithelial Na^+ channel (ENaC) subunits at the apical membrane (Alvarez De La Rosa *et al.*, 2002; Blazer-Yost *et al.*, 1998; Lang *et al.*, 2009; Loffing *et al.*, 2009; Soundararajan *et al.*, 2009; Soundararajan *et al.*, 2007; Soundararajan *et al.*, 2005). The MR-SGK1-ENaC pathway is critical to the long-term regulation of blood pressure and many drugs used to treat hypertension (ACE inhibitors, MR antagonists) promote diuresis / natriuresis by disrupting this mechanism. It is therefore interesting that KDAC inhibitors (KDACi) promote acetylation of heterologously expressed MR and reduce the transcriptional activity of this protein (Lee *et al.*, 2013). Moreover, KDACi also suppress aldosterone-induced gene transcripts in human embryonic kidney (HEK) cells, apparently by promoting acetylation of the endogenous MR (Lee *et al.*, 2013). Furthermore, such substances also block the development of hypertension in unilaterally nephrectomised mice exposed to a high salt diet and injected with DOCA (deoxycorticosterone acetate), an MR agonist (Lee *et al.*, 2013).

Whilst this work suggests KDACi might lower blood pressure by suppressing aldosterone-induced Na^+ retention (Lee *et al.*, 2013), it is important to note that HEK cells, although

derived from renal tissue, do not display a phenotype representative of any nephron segment. Indeed, these cells express voltage-gated Na^+ channels suggesting that they display a neural, rather than an epithelial phenotype (Mansell *et al.*, 2011; Moran *et al.*, 2000). Although aldosterone did induce gene expression in these cells (Lee *et al.*, 2013), the effect upon SGK1 developed over ~24 h (Lee *et al.*, 2013) and this extremely slow response is in stark contrast with data from CCD cells where hormone-induced Na^+ transport is apparent after only ~1 h. This relatively rapid response is accompanied by large (~40 fold) increases in the abundance of SGK1 mRNA and protein, and a clear increase in cellular SGK1 activity (Lang *et al.*, 2009; Lang *et al.*, 2006; Mansley *et al.*, 2016). It is therefore difficult to relate the effects of KDACi on HEK cells to events within the CCD. In order to test the hypothesis that KDAC inhibitors are able to suppress the MR-mediated stimulation of Na^+ absorption in the CCD, we have now explored the effects of Trichostatin A (TSA), a pan-KDACi, upon the responses to physiologically relevant concentrations of aldosterone in mouse CCD cells ($mCCD_{cl1}$).

Materials and methods

Electrophysiological measurements

Standard methods were used to maintain murine $mCCD_{cl1}$ cells in serial culture (Mansley *et al.*, 2015) and experiments undertaken using confluent cells (7 – 8 days culture) that had been deprived of hormones / growth factors for ~48 h (Gaeggeler *et al.*, 2005; Mansley *et al.*, 2015). ENaC-mediated Na^+ transport was usually quantified in cells grown on Transwell membranes by measuring transepithelial voltage (V_t) and resistance (R_t) using an epithelial volt-ohm-meter (WPI EVOM^2, Hitchin, Herts., UK). Some experiments were undertaken using cells grown on Costar Snapwell membranes that were mounted in Ussing chambers where V_t and R_t were monitored continuously (Mansley *et al.*, 2010b). Equivalent short circuit current (I_{Eq}) was then calculated from Ohm's Law (*i.e.* V_t / R_t). All electrometric experiments were terminated by adding amiloride (10 μM), an ENaC blocker, to the solution bathing the apical side of the membrane. The small current (~5% of control) that persisted under these conditions was then subtracted from the previously measured values of I_{Eq} to quantify the transepithelial Na^+ current flowing *via* ENaC (I_{Amil}). Since V_t was expressed relative to an earth electrode in the basolateral bath, the absorption of Na^+ moving from the apical to the basal compartment will generate negative current.

Western analysis of extracted protein

Confluent cells were washed three times with ice cold phosphate-buffered saline before being scraped into ice cold lysis buffer containing protease and phosphatase inhibitors (Mansley *et al.*, 2010b). The cellular lysates were then ultrasonicated to ensure disruption, centrifuged to remove insoluble debris and their protein contents determined (Bradford reagent, Bio-Rad, Hertfordshire, UK). Aliquots of extracted protein (40 μg) were then fractionated on sodium dodecyl sulphate – polyacrylamide gels and transferred to Hybond PVDF membranes that were probed using antibodies detailed below. Immunoreactive proteins were detected using peroxide-conjugated secondary antibodies / enhanced chemiluminescence (ECL). Once this analysis was complete, gels were stripped and then re-probed using antibodies against β-actin or total NDRG1 to provide markers of protein loading. Fuller details are provided elsewhere (Mansley *et al.*, 2016; Mansley *et al.*, 2010b).

Protein samples derived from each experiment were processed in parallel and analysed on the same gels. This was important since Western analysis cannot produce entirely uniform data and ensuring that samples from entire experiments were analysed together therefore guarantees that variations between batches of antibody, ECL reagents, efficiencies of protein transfer to blotting membranes etc. do not contribute to the observed variation within each experimental group. It is evident from the presented images that some of the antibodies used identified additional bands in certain experiments. These additional bands were unaltered by any experimental manoeuvres and were therefore assumed to be irrelevant to the present study and ignored in our analyses. To derive quantitative data, high resolution, uncompressed digital images (TIF format) of each developed blot were obtained using a Chemi DOC MP Imaging System (BioRad, Hemel Hemptead, Herts. UK). The optical densities of all relevant bands were measured digitally (ImageJ, RRID:SCR_003070) and the data from each series of experiments collated. The mean optical density associated with protein extracted from cells deprived of hormones throughout the entire experimental period (i.e. control) was then determined. The individual data points from the entire series of experiments, whether from control or experimental cells, were then normalised to this overall mean value. The results of all such experiments are therefore expressed relative to a mean control signal. The effects of TSA upon the abundance of each acetylated protein were quantified densitometrically using ImageJ (Rueden *et al.*, 2017) using the expression $OD_{TSA} - {}_{Control} / OD_{Max} - OD_{Control}$. In this expression OD_{TSA} refers to the optical density quantified in cells exposed to a particular concentration of TSA, whist $OD_{Control}$ and OD_{Max} respectively refer to the corresponding values from control cells (*i.e.* cells simply exposed to solvent vehicle) and cells exposed to a

maximally-effective concentration of TSA. The results of this analysis are plotted (Fractional response, mean ± 95% CI) against the concentration of TSA. The presented images show individual gels judged to represent the results obtained in the entire series of experiments. None of these images were manipulated in such a way to conceal, move or introduce any specific feature. Cited molecular weights were estimated by reference to standards that were run on each gel.

Data and Statistical analysis

The effects of TSA upon responses to hormones were explored using strictly paired protocols in which control cells were exposed to a relevant concentration of the appropriate solvent vehicle. For all experiments, cells were grown on filters matched by: passage number, date of thaw, seeding density and culture conditions. For experiments, each matched filter of cells was randomly allocated to an experimental group, and values of *n* refer to the number of times the entire protocol was repeated. Pooled data are shown as mean ± the 95% confidence interval (95% CI) and the results of individual experiments are shown in all figures. Statistical analysis was undertaken using GraphPad Prism 7.03 (Graph Pad Software Ltd., San Diego, CA, USA. RRID:SCR_002798). Where appropriate, the pooled data derived from each set of experiments were firstly analysed by 2 way ANOVA. If this analysis identified statistically significant variations between the experimental groups, we undertook further analysis using Tukey's post hoc test with correction for multiple comparisons in order to identify differences between particular experimental groups. Values of $P < 0.05$ were considered to indicate statistical significance. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Experimental materials

Amiloride, aldosterone, insulin and general laboratory reagents were from Sigma (Poole, Dorset, UK). Antibodies against the $Thr^{346/356/366}$-phosphorylated and total forms of the protein encoded by n-myc downstream regulated gene 1 (NDRG1) and against SGK1 were purchased from the MRC Protein Phosphorylation Unit, University of Dundee (Dundee, UK). The antibodies against β-actin (RRID:AB_476697) and acetylated α-tubulin (RRID:AB_609894) were from Sigma (Poole, Dorset, UK), the antibodies against acetylated histone H3 (RRID:AB_823528) and H4 (RRID:AB_2448400) were from Cell Signalling Technologies (Leiden, The Netherlands).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a; Alexander *et al.*, 2017b; Alexander *et al.*, 2017c).

Results

The response of CCD cells to aldosterone

Table 1 shows the results of electrometric experiments that explored the effects of aldosterone (3 nM, 3 h) on cells grown on Transwell membranes. Aldosterone hyperpolarized V_t and reduced R_t and analysis using Ohm's Law revealed a clear increase in the magnitude of I_{Eq} (Table 1). Subsequently exposing unstimulated and aldosterone-stimulated cells to apical amiloride (10 μM), an ENaC blocker, depolarized V_t, increased R_t and caused >90% block of I_{Eq} (Table 1). Whilst these findings confirm that I_{Eq} is due, almost exclusively, to electrogenic Na^+ absorption *via* ENaC, it is interesting that aldosterone did augment the small current that persisted in the presence of amiloride (Table 1). The physiological basis of this small, amiloride resistant current was not investigated, but it is possible that it reflects the secretion of Cl^- and / or $HCO3^-$ across the apical membrane. To complete the analysis of these data, we quantified I_{Amil} (see Methods) and our data show clearly that aldosterone causes a 2.7 ± 0.2 fold increase (mean ± 95% CI) in the magnitude of this current (unstimulated: -7.5 ± 1.3 μA cm^{-2}; aldosterone-stimulated: -20.2 ± 3.0 μA cm^{-2}, n = 12, mean ± 95% CI, $P < 0.05$ Student's unpaired t test).

TSA-induced acetylation of cellular proteins

TSA was one of the first KDAC inhibitors to be described (Yoshida *et al.*, 1990) and is widely used as a reference in research within this field. It has been used to determine anti-tumour activity in pre-clinical models of cancer but it has not been assessed in a clinical setting (Sanderson *et al.*, 2004). Studies of protein extracted from hormone-deprived cells exposed to TSA (0.01 – 100 μM) and / or solvent vehicle for 6 h showed that this pan-KDACi increased the abundance of acetylated histone H3 (Fig 1A), histone H4 (Fig 1B) and α-tubulin (Fig 1C). Analysis of sigmoid curves fitted to these data by least squares regression (Fig 1A – C) showed that half maximal responses occurred at ~0.15 μM (histone H3: EC_{50} =

0.14 ± 0.07 μM; histone H4: EC_{50} = 0.15 ± 0.19 μM; α-tubulin: EC_{50} = 0.20 μM ± 0.13 μM) and established that concentrations of TSA > 1 μM were maximally effective (Fig 1A – C). Parallel analyses using an antibody against β-actin confirmed that identical amounts of protein had been loaded onto each gel (Fig 1D) and we therefore conclude that exposure to TSA promotes acetylation of all three proteins. Fig 2 shows that the effects on histone H3 and H4 became apparent after ~1 h and reached plateau values after 6 – 12 h (Fig 2A, B). The acetylation of α-tubulin, on the other hand, peaked at ~1 h (Fig 2C). Although the effects on all three proteins declined throughout the remainder of the experimental period, increased acetylation persisted until at least 24 h (Fig 2A – C). Parallel analyses using the β-actin antibody confirmed that essentially identical amounts of cellular protein had been loaded onto each gel (Fig. 2D).

TSA disrupts the electrometric response to aldosterone

We explored the effects of TSA (1 μM) upon the electrometric response to aldosterone using the experimental protocol shown in Fig 3A. Measurements made at the onset of these experiments showed that I_{Eq} was normally ~8 μA cm^{-2} (Fig 3B) and this parameter was unaffected by exposure (2 h) to 1 μM TSA (Fig 3C). Control and TSA-treated cells were then exposed to 3 nM aldosterone and / or solvent vehicle and the electrometric measurements repeated after a further 3 h. Whilst the control data confirmed that aldosterone normally augments I_{Eq} (Fig 3D), this hormone had only very small effects on TSA-treated cells (Fig 3D). Further analysis of these data confirmed (Mansley *et al.*, 2015) that aldosterone normally augments I_{Amil} (Fig 3F). Although a small response was seen in the TSA-treated cells, its magnitude was only 15.7 ± 1.0 % of control (Fig 3) and therefore we conclude that TSA causes substantial (~85%) loss of sensitivity to aldosterone.

The control of cellular SGK1 abundance / activity

Cellular SGK1 activity was assessed using an established method that is based upon the identification of residues within an endogenous protein (NDRG1-$Thr^{346/356/366}$) that are phosphorylated by SGK1 but not by other, closely related kinases (Inglis *et al.*, 2009; Murray *et al.*, 2004). Once the electrometric measurements described above were completed, protein was extracted from cells for Western analysis so that we could correlate the electrometric data with changes to the phosphorylation status of these residues. These experiments showed clearly that the aldosterone-induced augmentation of I_{Amil} was associated with an increase in the abundance of $Thr^{346/356/366}$-phosphorylated NDRG1 (Fig 4A) that occurred without change

to the overall NDRG1 expression level (Fig 4A). Aldosterone thus promotes phosphorylation of NDRG1-Thr$^{346/356/366}$ and this finding confirms (Inglis *et al.*, 2009; Murray *et al.*, 2004) that this hormone normally increases cellular SGK1 activity. No such response was seen in TSA-treated cells (Fig 4). Parallel analyses using an antibody against SGK1 itself showed that aldosterone also increased the abundance of SGK1 protein and analyses undertaken using the β-actin antibody confirmed that this effect could not be attributed to variations in the mass of protein loaded onto the gels (Fig 4B). Aldosterone therefore increases the abundance of this protein and this response, in common with the associated increase in cellular SGK1 activity, was abolished by TSA (Fig 4B).

Effects of TSA upon the response of CCD cells to insulin

Fig 5A shows continuous records of I_{Amil} derived from cells mounted in Ussing chambers and acutely exposed to insulin (20 nM). As anticipated (Faletti *et al.*, 2002; Gonzalez-Rodriguez *et al.*, 2007; Mansley *et al.*, 2016; Mansley *et al.*, 2010b; Record *et al.*, 1998), insulin caused a clear and rapid augmentation of I_{Amil} although the response ($\Delta I_{Amil} \sim 11$ μA cm^{-2}, Fig 5A) was smaller than the response to aldosterone. Fig 5A also includes data from experiments undertaken using age-matched cells pre-treated (2 h) with 1 μM TSA. The responses seen in these cells were essentially identical to control indicating that TSA does not modify the electrometric response to this hormone. The insulin-induced augmentation of I_{Amil} was associated with an increase in the abundance of Thr$^{346/356/366}$-phosphorylated NDRG1 (Fig 5B) that occurred with no change to the overall NDRG1 expression level (Fig 5B). These data accord with previous findings (Mansley *et al.*, 2010a; Mansley *et al.*, 2010b) and therefore confirm that insulin normally activates SGK1 in CCD cells. This response persisted in the presence of TSA (1 μM), a novel finding which shows that insulin can still activate SGK1 following KDAC inhibition.

Discussion

Design of the present study

The controlled reabsorption of Na^+ within the CCD is critical to blood pressure homeostasis and depends upon ENaC, a Na^+-selective channel present in the apical membrane of principal cells in the CCD. In unstimulated cells the amount of ENaC in this membrane is restricted by the continual internalisation of channel subunits and this internalisation process limits the Na^+ permeability of the apical membrane and restricts the amount of Na^+ that can be recovered from the tubular fluid. Aldosterone promotes Na^+ retention by binding to the MR and

inducing expression of several genes, including that encoding SGK1 (Lang *et al.*, 2006). Although other mechanisms may well be involved (Frindt *et al.*, 2016; Soundararajan *et al.*, 2012), a substantial body of work shows that increased cellular SGK1 activity can inhibit this ENaC internalisation mechanism and thus allow the Na^+ permeability of the apical membrane to rise. This, in turn, increases the amount of Na^+ recovered from the tubular fluid (Debonneville *et al.*, 2001; Snyder, 2005; Snyder *et al.*, 2004). Earlier studies of cultured CCD cells showed that the natriuretic responses to aldosterone and insulin were abolished by pharmacological inhibition of SGK1 (Mansley *et al.*, 2018; Mansley *et al.*, 2010b), and this cannot be attributed to a nonspecific effect since the responses to peptide hormones that signal *via* cAMP / PKA are preserved when signalling *via* SGK1 is blocked. Hormone-induced changes to cellular SGK1 activity are therefore central to the regulation of Na^+ retention.

Administration of KDACi was shown to abolish the high blood pressure seen in a mouse model of hypertension, an observation attributed to loss of MR function (Lee *et al.*, 2013). This therefore implied that KDAC inhibition would abolish MR-mediated activation of the SGK1-ENaC pathway in the CCD. The aim of the present study was to test this hypothesis using a clinically applicable *in vitro* model of CCD cells, the $mCCD_{cl1}$ cell line. Our initial experiments were thereby focused on characterisation of the control response of these cells to aldosterone. Relatively brief (3 h) exposure of these cells to this hormone clearly augmented ENaC-mediated Na^+ transport and so, in contrast to HEK cells (Lee *et al.*, 2013), $mCCD_{cl1}$ cells display a relatively rapid response to aldosterone. Since the concentration of aldosterone used (3 nM) approximates to the circulating concentration in salt-deprived mice (Bertog *et al.*, 2008; Gaeggeler *et al.*, 2005), these data also confirm that $mCCD_{cl1}$ cells are sensitive to physiologically-relevant concentrations of this hormone (Mansley *et al.*, 2018). This point is significant to the present study since high concentrations of aldosterone (> ~10 nM) activate the GR as well as MR (Gaeggeler *et al.*, 2005). We estimate (Gaeggeler *et al.*, 2005) that 3 nM aldosterone will provide essentially complete (>~90%) occupancy of the MR but negligible binding to the GR, and therefore attribute the present responses to MR-mediated stimulation of Na^+ transport *via* ENaC.

Analysis of protein extracted from TSA-treated cells revealed clear increases in the abundance of acetylated histone H3 and H4, these are nuclear proteins that are archetypical class I KDAC substrates demonstrated in several cell types (Bantscheff *et al.*, 2011; Vigushin *et al.*, 2001; Yoshida *et al.*, 1995). In addition to nuclear proteins, TSA treatment also

increased abundance of acetylated α-tubulin in $mCCD_{cl1}$ cells, a protein target of class II KDACs. Moreover, whilst the acetylation of nuclear histone H3 / H4 proteins developed over ~6 h, the acetylation of α-tubulin developed over a much shorter time period (~1 h). This observation supports the reported mechanistic basis of KDAC activity and the requirement for class I KDACs to translocate to the nuclear compartment to exert their effect upon histones, as opposed to cytoplasmic proteins which can be directly modified.

The ability of the pan-KDACi TSA to modify both cytoplasmic and nuclear proteins, in combination with our earlier studies, confirming a physiologically relevant response of the $mCCD_{cl1}$ cells to MR activation, allowed us to thereby design a protocol to test the theory that KDACi suppress MR-mediated activation of the SGK1-ENaC pathway in CCD cells. Aldosterone normally increased the magnitude of I_{Amil} by ~2.5 fold and the 95% confidence interval associated with this response was ~0.2 fold. This implies that the smallest inhibitory action detectible by our study is ~20%. However, the previously reported animal studies had indicated that administration of KDACi essentially abolished experimental hypertension (Lee *et al.*, 2013) which, if correctly attributed to loss of MR function, would predict that KDAC inhibition associates with substantial inhibition of the response to aldosterone. Our experimental protocol therefore has sufficient statistical power to allow us to properly evaluate this hypothesis.

TSA supresses the response to aldosterone without directly affecting SGK1–ENaC

TSA caused substantial (~85%) inhibition of the electrometric response to aldosterone and abolished the associated increases in the abundance and activity of SGK1. These findings are consistent with the hypothesis that inhibition of KDACs cause loss of MR function (Lee *et al.*, 2013). Although aldosterone is the principal regulator of ENaC in the CCD, insulin also promotes Na^+ absorption *via* a mechanism dependent on the SGK1-ENaC signalling pathway (Blazer-Yost *et al.*, 2003; Blazer-Yost *et al.*, 1998; Blazer-Yost *et al.*, 1992; Faletti *et al.*, 2002; Mansley *et al.*, 2016; Mansley *et al.*, 2010a; Pearce *et al.*, 2007; Record *et al.*, 1998; Wang *et al.*, 2001). However, despite both hormones increasing cellular SGK1 activity, TSA did not perturb the response to insulin. In contrast to aldosterone, insulin activates SGK1 without increasing the abundance of SGK1 protein or mRNA. Instead, this hormone acts *via* a non-genomic mechanism dependent upon PI3K (Cohen, 2006) an enzyme that increases the catalytic activity of SGK1 protein by controlling its phosphorylation (Kobayashi *et al.*, 1999; Park *et al.*, 1999). TSA therefore suppresses MR-mediated activation of the SGK1-ENaC

pathway by disrupting control over SGK1 protein abundance without affecting the PI3K-dependent, post-translational control of this pathway.

The mechanism that allows aldosterone to regulate ENaC-mediated Na^+ retention is complex and incompletely understood. However, it is now clear that the acetylation status of cellular proteins has a direct bearing upon several components of this pathway. For example, KDAC inhibition has been shown to promote acetylation of heat shock protein 90 (hsp90), a protein that controls the translocation of activated steroid receptors to the nucleus (Barnes, 2013; Jimenez-Canino *et al.*, 2016). Moreover, in contrast to the studies of wild type / mutant forms of the MR (Lee *et al.*, 2013), this works indicates that KDAC inhibitors do not alter the transcriptional activity of the receptor (Jimenez-Canino *et al.*, 2016). The inhibition of MR signalling which we now report may therefore be due, at least in part, to altered translocation of the MR between the cytoplasm and the nucleus (Jimenez-Canino *et al.*, 2016). However, KDAC inhibition has also been shown to promote acetylation of ENaC itself (Butler *et al.*, 2015). This modification seems to block the ENaC internalisation process that appears to limit Na^+ transport in unstimulated cells (Blazer-Yost *et al.*, 2005; Gonzalez-Rodriguez *et al.*, 2007; Wang *et al.*, 2001). Rather than supressing Na^+ absorption, these data therefore suggest that ENaC acetylation will augment Na^+ retention by increasing the surface abundance of the channel (Butler *et al.*, 2015). Whilst we cannot exclude the possibility that this process may augment Na^+ retention under certain conditions (Butler *et al.*, 2015), our data show that TSA has no effect on basal Na^+ transport and does not modify the response to insulin. It thus appears that inhibition of KDAC does not directly modify ENaC function under the present conditions.

The KDAC enzyme family contain at least 8 members (KDAC1 – 8) and it is now clear that different KDACs can fulfil different physiological roles. The acetylation status of the MR, for example, seems to be maintained by KDAC3 (Lee *et al.*, 2013), whilst KDAC6 and KDAC5 are respectively thought to determine the acetylation status of hsp90 (Jimenez-Canino *et al.*, 2016) and ENaC (Butler *et al.*, 2015). TSA is a broad spectrum KDAC inhibitor, and thus has the potential to interfere with all of these events. Moreover, although selective inhibitors are becoming available, their use is not straightforward since the KDAC isoforms are known interact with each other. For example, although acetylated α-tubulin is not a substrate for KDAC3, MI192, a highly selective inhibitor of KDAC3 (Boissinot *et al.*, 2012), promotes acetylation of this protein *via* an indirect mechanism that is ultimately dependent upon KDAC6 (Bacon *et al.*, 2015).

Conclusions and implications

TSA promoted the acetylation of nuclear (histone H3, H4) and cytoplasmic (α-tubulin) proteins indicating clear inhibition of KDAC. Moreover, TSA also blocked the aldosterone-dependent control over ENaC-mediated Na^+ transport and cellular SGK1 activity, but did not affect the corresponding responses to insulin. The present data therefore show that TSA selectively disrupts the regulation of Na^+ transport *via* genomic mechanisms. These data accord with studies of the heterologously expressed MR (Lee *et al.*, 2013) and establish a physiological basis for the antihypertensive actions seen *in vivo* (Lee *et al.*, 2013). KDAC inhibition may therefore provide a novel means of lowering blood pressure in hypertensive patients and it is therefore important to fully define the mechanisms that allow these compounds to act in this way. The effects described in this earlier study (Lee *et al.*, 2013) were attributed to increased acetylation of the MR itself and, although we did not directly monitor the acetylation status of this receptor, MR acetylation could well explain the inhibitory action of TSA described here. However, it is important to stress that genomic responses to steroids such as aldosterone occur *via* complex mechanisms that are still incompletely understood. In this context it is interesting that inhibition of KDAC5/6 appears to suppress the translocation of the activated MR from the cytoplasm into the nucleus without altering the transcriptional activity of the receptor itself (Jimenez-Canino *et al.*, 2016). We cannot exclude the possibility that such a mechanism may contribute to the effect of TSA which we now report and it is also possible that the response may reflect changes to the acetylation of histone itself. Future studies, in which the acetylation status of the MR along with other physiologically important proteins are critically assessed, will therefore be required to establish the mechanism that allows TSA to suppress aldosterone-induced Na^+ transport.

Declaration of Transparency and Scientific Rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Immunoblotting and Immunochemistry, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Table 1: The electrometric response to aldosterone

	V_t (mV)			R_t (Ω cm^{-2})			I_{eq} (μA cm^{-2})		
	Baseline	3h treatment	Amil.	Baseline	3h treatment	Amil.	Baseline	3h treatment	Amil.
Vehicle	-17.2 ± 5.9	-16.3 ± 5.1	0.5 ± 0.7Ŧ	2105 ± 342	2125 ± 363	3340 ± 650 Ŧ	-7.8 ± 1.6	-7.4 ± 1.4	0.1 ± 0.2 Ŧ
Aldo.	-18.1 ± 5.2	-36.7 ± 7.4*	-5.9 ± 3.2* Ŧ	2277 ± 257	1666 ± 113*	3261 ± 461 Ŧ	-7.7 ± 1.5	-5.9 ± 3.2*	-1.6 ± 0.8* Ŧ

Electrometric measurements (V_t and R_t) were recorded, allowing calculation of equivalent short circuit current (I_{eq}), from cells grown on filters under control (vehicle) or aldosterone-treated conditions. Measurements were made: before treatment (baseline); 3 h after treatment (3h treatment) with solvent vehicle or aldosterone (Aldo, 3 nM); and 5 min after exposure to amiloride (Amil., 10 μM). Data are mean ± 95% confidence interval (n = 12), statistical significance was determined using a repeated-measures two-way ANOVA. Where appropriate, multiple comparisons were determined by Tukey's post-hoc test and significance has been denoted for comparisons between vehicle and aldosterone-treated cells (*) or comparisons of 3h treatment and amiloride (Ŧ).

660

661 **Figure Legends**

662 **Fig 1. TSA-induced acetylation of cellular proteins.** Cells were exposed to TSA (0.01 –
663 100 μM) and / or solvent vehicle for 6 h. Upper panels show representative blots probed with
664 acetylated histone H3 (A, Ac-Histone H3), histone H4 (B, Ac – Histone H4) and α-tubulin
665 (C, Ac-α-tubulin). All blots were reanalysed using an antibody against β-actin (D).
666 Molecular weight markers are marked and arrows denote relevant bands. Lower panels show
667 the densitometric analyses of the data derived from the entire series of experiments (n = 5).
668 The solid curves are sigmoid curves fitted to these data by least squares regression whilst the
669 vertical bars are centred on the mean values and show the 95% confidence interval. The mean
670 densitometric values of β-actin are expressed relative to the mean value from cells treated
671 with solvent vehicle of TSA.

672 **Fig 2. Time course of TSA-induced acetylation.** Protein extracted from cells exposed to
673 1 μM TSA for 0 – 24 h was subject to Western analysis using antibodies against the
674 acetylated forms of histone H3 (A, Ac-Histone H3, n = 6), histone H4 (B, Ac – Histone H4, n
675 = 6) and α-tubulin (C, Ac-α-tubulin, n = 6). All blots were then re-probed using the antibody
676 against β-actin (D). In each figure the upper panel shows the results of a representative
677 experiment whilst the densitometric analyses presented below show the changes to the
678 abundance of each protein plotted against time. Data are mean ± 95% CI.

679 **Fig 3. Effects of TSA upon the electrometric response to aldosterone.** (A) Each
680 horizontal bar represents a separate cultured epithelial layer and cells were exposed to TSA,
681 aldosterone and amiloride as indicated by the different shading. Electrometric data were
682 recorded (arrows) at the onset of each experiment (B); after 2 h exposure to TSA (1 μM) and
683 /or solvent vehicle (C); after the cells had been exposed to aldosterone (3 nM) and / or
684 solvent vehicle for a further 3 h (D), and after a final application of 10 μM apical amiloride
685 (E). Shading of the vertical bars (B-D) corresponds with those used in (A) to differentiate the
686 various stages of the experiment. Vertical columns show the mean values ± 95% CI (n = 7)
687 for each experimental group. Statistical significance was determined by repeated measures
688 two-way ANOVA and, where appropriate, multiple comparisons were determined by
689 Tukey's post-hoc test. (F) The amiloride-sensitive component of the transepithelial
690 equivalent current (I_{Amil}, mean ± 95% CI, n = 7) was quantified. Statistical significance was
691 determined by two-way ANOVA and, where appropriate, multiple comparisons were
692 determined by Tukey's post-hoc test.

Fig. 4. Effects of TSA upon aldosterone-induced SGK1 activity and abundance. (A) Typical blots obtained using antibodies against the $Thr^{346/356/366}$-phosphorylated form of the protein encoded by N-myc downstream regulated gene 1 (upper panel, NDRG1-P-$Thr^{346/356/366}$) and the equivalent full length protein (lower panel, T-NDRG1). (B) Typical blots obtained using antibodies against the serum and glucocorticoid-inducible kinase 1 (upper panel, T-SGK1) and β-actin (lower panel). Pooled data (right-hand panels) from the entire series of experiments showing (mean ± 95% CI, n = 5) the effects of aldosterone (3 nM, 3 h) upon the abundance of (A) NDRG1-P-$Thr^{346/356/366}$ (upper) and total NDRG1 (lower) or (B) SGK1 (upper) and b-actin (lower) in control and TSA-treated (1 μM , 2 h) cells. Statistical significance was determined by two-way ANOVA and, where appropriate, multiple comparisons were determined by Tukey's post-hoc test.

Fig. 5. Insulin-induced Na^+ transport and SGK1 activity. (A) Confluent cells on Costar Snapwell membranes were mounted in Ussing chambers so that the effects of insulin (20 nM) upon I_{Amil} could be continuously recorded (mean ± 95% CI, n = 6) under control conditions (left panel) and in cells that had been pre-treated (2 h) with 1 μM TSA (middle panel). The amiloride-sensitive responses to insulin (ΔI_{Amil}) were quantified and presented as mean ± 95% CI (right panel). (B) Typical blots obtained using antibodies against the $Thr^{346/356/366}$-phosphorylated form of the protein encoded by N-myc downstream regulated gene 1 (upper panel, NDRG1-P-$Thr^{346/356/366}$) and the equivalent full length protein (lower panel, T-NDRG1). Pooled data (right-hand panels) from the entire series of experiments showing (mean ± 95% CI, n = 6) the effects of insulin (20 nM, 1 h) upon the abundance of NDRG1-P-$Thr^{346/356/366}$ (upper) and total NDRG1 (lower) in control and TSA-treated (1 μM , 2 h) cells. Statistical significance was determined by two-way ANOVA and, where appropriate, multiple comparisons were determined by Tukey's post-hoc test.