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

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1
2 **TRICHOSTATIN A BLOCKS ALDOSTERONE-INDUCED Na⁺ TRANSPORT AND**
3 **CONTROL OF SERUM- AND GLUCOCORTICOID-INDUCIBLE KINASE 1 IN**
4 **CORTICAL COLLECTING DUCT CELLS**
5

6 Running title: KDAC inhibition and ENaC activity
7

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18
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20 MKM, AJR, and SLF performed experiments; SMW, MAB, JHG, MKM, AJR and SLF
21 interpreted results of experiments; SMW, MKM, AJR and SLF prepared figures; SMW
22 drafted manuscript; SMW, MKM, MAB and JHG edited and revised manuscript; SMW,
23 MAB, JHG, MKM, AJR and SLF approved final version of the manuscript.
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26

27 **Abstract**

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

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

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

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

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

50 **Abbreviations**

51	CCD	Cortical collecting duct
52	DOCA	Deoxycorticosterone acetate
53	ENaC	Epithelial Na ⁺ channel
54	HDAC	Histone deacetylase
55	HEK	Human embryonic kidney
56	I_{amil}	Amiloride-sensitive current

57	I_{eq}	Equivalent short circuit current
58	KAT	Lysine acetyl transferase
59	KDAC	Lysine deacetylase
60	KDACi	Lysine deacetylase inhibitor
61	MR	Mineralocorticoid receptor
62	NDRG1	N-myc downstream regulated gene 1
63	GR	Glucocorticoid receptor
64	SGK1	Serum- and glucocorticoid-inducible kinase 1
65	R_t	Transepithelial resistance
66	TSA	Trichostatin A
67	V_t	Transepithelial voltage

68

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72 antibody.

73

74 Conflicts of interest: The authors report no conflicts of interest.

75

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

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84 **What is already known?**

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

88 **What this study adds**

- 89 • As anticipated, TSA consistently promoted acetylation of cytoplasmic and nuclear
90 proteins
91 • Trichostatin A (TSA) suppressed the MR-mediated transcription of *sgkl* and blocked
92 aldosterone-induced Na⁺ absorption

93 **Clinical significance**

- 94 • Hypertension can be treated with drugs that disrupt the response to aldosterone
95 • Agents that promote protein acetylation may provide a novel means of suppressing
96 aldosterone-induced Na⁺ retention

97

98

Introduction

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

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

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

131 derived from renal tissue, do not display a phenotype representative of any nephron segment.
132 Indeed, these cells express voltage-gated Na⁺ channels suggesting that they display a neural,
133 rather than an epithelial phenotype (Mansell *et al.*, 2011; Moran *et al.*, 2000). Although
134 aldosterone did induce gene expression in these cells (Lee *et al.*, 2013), the effect upon SGK1
135 developed over ~24 h (Lee *et al.*, 2013) and this extremely slow response is in stark contrast
136 with data from CCD cells where hormone-induced Na⁺ transport is apparent after only ~1 h.
137 This relatively rapid response is accompanied by large (~40 fold) increases in the abundance
138 of SGK1 mRNA and protein, and a clear increase in cellular SGK1 activity (Lang *et al.*,
139 2009; Lang *et al.*, 2006; Mansley *et al.*, 2016). It is therefore difficult to relate the effects of
140 KDACi on HEK cells to events within the CCD. In order to test the hypothesis that KDAC
141 inhibitors are able to suppress the MR-mediated stimulation of Na⁺ absorption in the CCD,
142 we have now explored the effects of [Trichostatin A](#) (TSA), a pan-KDACi, upon the responses
143 to physiologically relevant concentrations of aldosterone in mouse CCD cells (mCCD_{cl1}).

144

Materials and methods

145 Electrophysiological measurements

146 Standard methods were used to maintain murine mCCD_{cl1} cells in serial culture (Mansley *et al.*
147 *et al.*, 2015) and experiments undertaken using confluent cells (7 – 8 days culture) that had been
148 deprived of hormones / growth factors for ~48 h (Gaeggeler *et al.*, 2005; Mansley *et al.*,
149 2015). ENaC-mediated Na⁺ transport was usually quantified in cells grown on Transwell
150 membranes by measuring transepithelial voltage (V_t) and resistance (R_t) using an epithelial
151 volt-ohm-meter (WPI EVOM², Hitchin, Herts., UK). Some experiments were undertaken
152 using cells grown on Costar Snapwell membranes that were mounted in Ussing chambers
153 where V_t and R_t were monitored continuously (Mansley *et al.*, 2010b). Equivalent short
154 circuit current (I_{Eq}) was then calculated from Ohm's Law (*i.e.* V_t / R_t). All electrometric
155 experiments were terminated by adding amiloride (10 μ M), an ENaC blocker, to the solution
156 bathing the apical side of the membrane. The small current (~5% of control) that persisted
157 under these conditions was then subtracted from the previously measured values of I_{Eq} to
158 quantify the transepithelial Na⁺ current flowing *via* ENaC (I_{Amil}). Since V_t was expressed
159 relative to an earth electrode in the basolateral bath, the absorption of Na⁺ moving from the
160 apical to the basal compartment will generate negative current.

161 Western analysis of extracted protein

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

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

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

201 **Data and Statistical analysis**

202 The effects of TSA upon responses to hormones were explored using strictly paired protocols
203 in which control cells were exposed to a relevant concentration of the appropriate solvent
204 vehicle. For all experiments, cells were grown on filters matched by: passage number, date of
205 thaw, seeding density and culture conditions. For experiments, each matched filter of cells
206 was randomly allocated to an experimental group, and values of n refer to the number of
207 times the entire protocol was repeated. Pooled data are shown as mean \pm the 95% confidence
208 interval (95% CI) and the results of individual experiments are shown in all figures.
209 Statistical analysis was undertaken using GraphPad Prism 7.03 (Graph Pad Software Ltd.,
210 San Diego, CA, USA. RRID:SCR_002798). Where appropriate, the pooled data derived from
211 each set of experiments were firstly analysed by 2 way ANOVA. If this analysis identified
212 statistically significant variations between the experimental groups, we undertook further
213 analysis using Tukey's post hoc test with correction for multiple comparisons in order to
214 identify differences between particular experimental groups. Values of $P < 0.05$ were
215 considered to indicate statistical significance. The data and statistical analysis comply with
216 the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*,
217 2015).

218 **Experimental materials**

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

227

228 **Nomenclature of Targets and Ligands**

229 Key protein targets and ligands in this article are hyperlinked to corresponding entries in
 230 <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS
 231 Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the
 232 Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a; Alexander *et al.*,
 233 2017b; Alexander *et al.*, 2017c).

234

Results235 **The response of CCD cells to aldosterone**

236 Table 1 shows the results of electrometric experiments that explored the effects of
 237 aldosterone (3 nM, 3 h) on cells grown on Transwell membranes. Aldosterone hyperpolarized
 238 V_t and reduced R_t and analysis using Ohm's Law revealed a clear increase in the magnitude
 239 of I_{Eq} (Table 1). Subsequently exposing unstimulated and aldosterone-stimulated cells to
 240 apical amiloride (10 μ M), an ENaC blocker, depolarized V_t , increased R_t and caused >90%
 241 block of I_{Eq} (Table 1). Whilst these findings confirm that I_{Eq} is due, almost exclusively, to
 242 electrogenic Na^+ absorption *via* ENaC, it is interesting that aldosterone did augment the small
 243 current that persisted in the presence of amiloride (Table 1). The physiological basis of this
 244 small, amiloride resistant current was not investigated, but it is possible that it reflects the
 245 secretion of Cl^- and / or HCO_3^- across the apical membrane. To complete the analysis of
 246 these data, we quantified I_{Amil} (see Methods) and our data show clearly that aldosterone
 247 causes a 2.7 ± 0.2 fold increase (mean \pm 95% CI) in the magnitude of this current
 248 (unstimulated: $-7.5 \pm 1.3 \mu A cm^{-2}$; aldosterone-stimulated: $-20.2 \pm 3.0 \mu A cm^{-2}$, $n = 12$, mean
 249 \pm 95% CI, $P < 0.05$ Student's unpaired t test).

250 **TSA-induced acetylation of cellular proteins**

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

259 $0.14 \pm 0.07 \mu\text{M}$; histone H4: $\text{EC}_{50} = 0.15 \pm 0.19 \mu\text{M}$; α -tubulin: $\text{EC}_{50} = 0.20 \mu\text{M} \pm 0.13 \mu\text{M}$)
260 and established that concentrations of TSA $> 1 \mu\text{M}$ were maximally effective (Fig 1A – C).
261 Parallel analyses using an antibody against β -actin confirmed that identical amounts of
262 protein had been loaded onto each gel (Fig 1D) and we therefore conclude that exposure to
263 TSA promotes acetylation of all three proteins. Fig 2 shows that the effects on histone H3 and
264 H4 became apparent after ~ 1 h and reached plateau values after 6 – 12 h (Fig 2A, B). The
265 acetylation of α -tubulin, on the other hand, peaked at ~ 1 h (Fig 2C). Although the effects on
266 all three proteins declined throughout the remainder of the experimental period, increased
267 acetylation persisted until at least 24 h (Fig 2A – C). Parallel analyses using the β -actin
268 antibody confirmed that essentially identical amounts of cellular protein had been loaded onto
269 each gel (Fig. 2D).

270 **TSA disrupts the electrometric response to aldosterone**

271 We explored the effects of TSA ($1 \mu\text{M}$) upon the electrometric response to aldosterone using
272 the experimental protocol shown in Fig 3A. Measurements made at the onset of these
273 experiments showed that I_{Eq} was normally $\sim 8 \mu\text{A cm}^{-2}$ (Fig 3B) and this parameter was
274 unaffected by exposure (2 h) to $1 \mu\text{M}$ TSA (Fig 3C). Control and TSA-treated cells were then
275 exposed to 3 nM aldosterone and / or solvent vehicle and the electrometric measurements
276 repeated after a further 3 h. Whilst the control data confirmed that aldosterone normally
277 augments I_{Eq} (Fig 3D), this hormone had only very small effects on TSA-treated cells (Fig
278 3D). Further analysis of these data confirmed (Mansley *et al.*, 2015) that aldosterone
279 normally augments I_{Amil} (Fig 3F). Although a small response was seen in the TSA-treated
280 cells, its magnitude was only $15.7 \pm 1.0 \%$ of control (Fig 3) and therefore we conclude that
281 TSA causes substantial ($\sim 85\%$) loss of sensitivity to aldosterone.

282 **The control of cellular SGK1 abundance / activity**

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

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

300 **Effects of TSA upon the response of CCD cells to insulin**

301 Fig 5A shows continuous records of I_{Amil} derived from cells mounted in Ussing chambers and
 302 acutely exposed to insulin (20 nM). As anticipated (Faletti *et al.*, 2002; Gonzalez-Rodriguez
 303 *et al.*, 2007; Mansley *et al.*, 2016; Mansley *et al.*, 2010b; Record *et al.*, 1998), insulin caused
 304 a clear and rapid augmentation of I_{Amil} although the response ($\Delta I_{Amil} \sim 11 \mu A cm^{-2}$, Fig 5A)
 305 was smaller than the response to aldosterone. Fig 5A also includes data from experiments
 306 undertaken using age-matched cells pre-treated (2 h) with 1 μM TSA. The responses seen in
 307 these cells were essentially identical to control indicating that TSA does not modify the
 308 electrometric response to this hormone. The insulin-induced augmentation of I_{Amil} was
 309 associated with an increase in the abundance of Thr^{346/356/366}-phosphorylated NDRG1 (Fig
 310 5B) that occurred with no change to the overall NDRG1 expression level (Fig 5B). These
 311 data accord with previous findings (Mansley *et al.*, 2010a; Mansley *et al.*, 2010b) and
 312 therefore confirm that insulin normally activates SGK1 in CCD cells. This response persisted
 313 in the presence of TSA (1 μM), a novel finding which shows that insulin can still activate
 314 SGK1 following KDAC inhibition.

315 **Discussion**

316 **Design of the present study**

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

323 inducing expression of several genes, including that encoding SGK1 (Lang *et al.*, 2006).
324 Although other mechanisms may well be involved (Frindt *et al.*, 2016; Soundararajan *et al.*,
325 2012), a substantial body of work shows that increased cellular SGK1 activity can inhibit this
326 ENaC internalisation mechanism and thus allow the Na⁺ permeability of the apical membrane
327 to rise. This, in turn, increases the amount of Na⁺ recovered from the tubular fluid
328 (Debonneville *et al.*, 2001; Snyder, 2005; Snyder *et al.*, 2004). Earlier studies of cultured
329 CCD cells showed that the natriuretic responses to aldosterone and insulin were abolished by
330 pharmacological inhibition of SGK1 (Mansley *et al.*, 2018; Mansley *et al.*, 2010b), and this
331 cannot be attributed to a nonspecific effect since the responses to peptide hormones that
332 signal *via* cAMP / PKA are preserved when signalling *via* SGK1 is blocked. Hormone-
333 induced changes to cellular SGK1 activity are therefore central to the regulation of Na⁺
334 retention.

335 Administration of KDACi was shown to abolish the high blood pressure seen in a mouse
336 model of hypertension, an observation attributed to loss of MR function (Lee *et al.*, 2013).
337 This therefore implied that KDAC inhibition would abolish MR-mediated activation of the
338 SGK1-ENaC pathway in the CCD. The aim of the present study was to test this hypothesis
339 using a clinically applicable *in vitro* model of CCD cells, the mCCD_{c11} cell line. Our initial
340 experiments were thereby focused on characterisation of the control response of these cells to
341 aldosterone. Relatively brief (3 h) exposure of these cells to this hormone clearly augmented
342 ENaC-mediated Na⁺ transport and so, in contrast to HEK cells (Lee *et al.*, 2013), mCCD_{c11}
343 cells display a relatively rapid response to aldosterone. Since the concentration of aldosterone
344 used (3 nM) approximates to the circulating concentration in salt-deprived mice (Bertog *et*
345 *al.*, 2008; Gaeggeler *et al.*, 2005), these data also confirm that mCCD_{c11} cells are sensitive to
346 physiologically-relevant concentrations of this hormone (Mansley *et al.*, 2018). This point is
347 significant to the present study since high concentrations of aldosterone (> ~10 nM) activate
348 the GR as well as MR (Gaeggeler *et al.*, 2005). We estimate (Gaeggeler *et al.*, 2005) that 3
349 nM aldosterone will provide essentially complete (>~90%) occupancy of the MR but
350 negligible binding to the GR, and therefore attribute the present responses to MR-mediated
351 stimulation of Na⁺ transport *via* ENaC.

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

356 increased abundance of acetylated α -tubulin in mCCD_{cl1} cells, a protein target of class II
357 KDACs. Moreover, whilst the acetylation of nuclear histone H3 / H4 proteins developed over
358 ~6 h, the acetylation of α -tubulin developed over a much shorter time period (~1 h). This
359 observation supports the reported mechanistic basis of KDAC activity and the requirement
360 for class I KDACs to translocate to the nuclear compartment to exert their effect upon
361 histones, as opposed to cytoplasmic proteins which can be directly modified.

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

374 **TSA suppresses the response to aldosterone without directly affecting SGK1–ENaC**

375 TSA caused substantial (~85%) inhibition of the electrometric response to aldosterone and
376 abolished the associated increases in the abundance and activity of SGK1. These findings are
377 consistent with the hypothesis that inhibition of KDACs cause loss of MR function (Lee *et al.*
378 *et al.*, 2013). Although aldosterone is the principal regulator of ENaC in the CCD, insulin also
379 promotes Na⁺ absorption *via* a mechanism dependent on the SGK1-ENaC signalling pathway
380 (Blazer-Yost *et al.*, 2003; Blazer-Yost *et al.*, 1998; Blazer-Yost *et al.*, 1992; Faletti *et al.*,
381 2002; Mansley *et al.*, 2016; Mansley *et al.*, 2010a; Pearce *et al.*, 2007; Record *et al.*, 1998;
382 Wang *et al.*, 2001). However, despite both hormones increasing cellular SGK1 activity, TSA
383 did not perturb the response to insulin. In contrast to aldosterone, insulin activates SGK1
384 without increasing the abundance of SGK1 protein or mRNA. Instead, this hormone acts *via*
385 a non-genomic mechanism dependent upon PI3K (Cohen, 2006) an enzyme that increases the
386 catalytic activity of SGK1 protein by controlling its phosphorylation (Kobayashi *et al.*, 1999;
387 Park *et al.*, 1999). TSA therefore suppresses MR-mediated activation of the SGK1-ENaC

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

390 The mechanism that allows aldosterone to regulate ENaC-mediated Na⁺ retention is complex
391 and incompletely understood. However, it is now clear that the acetylation status of cellular
392 proteins has a direct bearing upon several components of this pathway. For example, KDAC
393 inhibition has been shown to promote acetylation of heat shock protein 90 (hsp90), a protein
394 that controls the translocation of activated steroid receptors to the nucleus (Barnes, 2013;
395 Jimenez-Canino *et al.*, 2016). Moreover, in contrast to the studies of wild type / mutant forms
396 of the MR (Lee *et al.*, 2013), this work indicates that KDAC inhibitors do not alter the
397 transcriptional activity of the receptor (Jimenez-Canino *et al.*, 2016). The inhibition of MR
398 signalling which we now report may therefore be due, at least in part, to altered translocation
399 of the MR between the cytoplasm and the nucleus (Jimenez-Canino *et al.*, 2016). However,
400 KDAC inhibition has also been shown to promote acetylation of ENaC itself (Butler *et al.*,
401 2015). This modification seems to block the ENaC internalisation process that appears to
402 limit Na⁺ transport in unstimulated cells (Blazer-Yost *et al.*, 2005; Gonzalez-Rodriguez *et al.*,
403 2007; Wang *et al.*, 2001). Rather than suppressing Na⁺ absorption, these data therefore suggest
404 that ENaC acetylation will augment Na⁺ retention by increasing the surface abundance of the
405 channel (Butler *et al.*, 2015). Whilst we cannot exclude the possibility that this process may
406 augment Na⁺ retention under certain conditions (Butler *et al.*, 2015), our data show that TSA
407 has no effect on basal Na⁺ transport and does not modify the response to insulin. It thus
408 appears that inhibition of KDAC does not directly modify ENaC function under the present
409 conditions.

410 The KDAC enzyme family contain at least 8 members (KDAC1 – 8) and it is now clear that
411 different KDACs can fulfil different physiological roles. The acetylation status of the MR, for
412 example, seems to be maintained by KDAC3 (Lee *et al.*, 2013), whilst KDAC6 and KDAC5
413 are respectively thought to determine the acetylation status of hsp90 (Jimenez-Canino *et al.*,
414 2016) and ENaC (Butler *et al.*, 2015). TSA is a broad spectrum KDAC inhibitor, and thus has
415 the potential to interfere with all of these events. Moreover, although selective inhibitors are
416 becoming available, their use is not straightforward since the KDAC isoforms are known
417 interact with each other. For example, although acetylated α -tubulin is not a substrate for
418 KDAC3, MI192, a highly selective inhibitor of KDAC3 (Boissinot *et al.*, 2012), promotes
419 acetylation of this protein *via* an indirect mechanism that is ultimately dependent upon
420 KDAC6 (Bacon *et al.*, 2015).

421 **Conclusions and implications**

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

445

446 **Declaration of Transparency and Scientific Rigour**

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

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659

Table 1: The electrometric response to aldosterone

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

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 μ M). Data are mean \pm 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 (†).

660

661

Figure Legends

662 **Fig 1. TSA-induced acetylation of cellular proteins.** Cells were exposed to TSA (0.01 –
663 100 μ M) 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 μ M 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 \pm 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 μ M) 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 μ M 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 \pm 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_{Aml} , mean \pm 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.

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

704 **Fig. 5. Insulin-induced Na⁺ transport and SGK1 activity.** (A) Confluent cells on Costar
705 Snapwell membranes were mounted in Ussing chambers so that the effects of insulin (20 nM)
706 upon I_{Amil} could be continuously recorded (mean \pm 95% CI, $n = 6$) under control conditions
707 (left panel) and in cells that had been pre-treated (2 h) with 1 μ M TSA (middle panel). The
708 amiloride-sensitive responses to insulin (ΔI_{Amil}) were quantified and presented as mean \pm
709 95% CI (right panel). (B) Typical blots obtained using antibodies against the Thr^{346/356/366}-
710 phosphorylated form of the protein encoded by N-myc downstream regulated gene 1 (upper
711 panel, NDRG1-P-Thr^{346/356/366}) and the equivalent full length protein (lower panel, T-
712 NDRG1). Pooled data (right-hand panels) from the entire series of experiments showing
713 (mean \pm 95% CI, $n = 6$) the effects of insulin (20 nM, 1 h) upon the abundance of NDRG1-P-
714 Thr^{346/356/366} (upper) and total NDRG1 (lower) in control and TSA-treated (1 μ M, 2 h) cells.
715 Statistical significance was determined by two-way ANOVA and, where appropriate,
716 multiple comparisons were determined by Tukey's post-hoc test.