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Glucocorticoids can activate the α-ENaC gene promoter independently of SGK1

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The role of SGK1 (serum- and glucocorticoid-induced protein kinase 1) in the glucocorticoid induction of α-ENaC (epithelial Na^+ channel α subunit) gene transcription was explored by monitoring the transcriptional activity of a luciferase-linked, α-ENaC reporter gene construct (pGL3-KR1) expressed in H441 airway epithelial cells. Dexamethasone evoked a concentration-dependent (EC_{50} ~4 μM) increase in transcriptional activity dependent upon a glucocorticoid response element in the α-ENaC sequence. Although dexamethasone also activated endogenous SGK1, artificially increasing cellular SGK1 activity by expressing a constitutively active SGK1 mutant (SGK1-S422D) in hormone-deprived cells did not activate pGL3-KR1. Moreover, expression of catalytically inactive SGK1 (SGK1-K127A) suppressed the activation of endogenous SGK1 without affecting the transcriptional response to dexamethasone. Increasing cellular PI3K (phosphoinositide 3-kinase) activity by expressing a membrane-anchored form of the catalytic PI3K-P110α subunit [CD2 (cluster of differentiation 2)-P110α] also activated endogenous SGK1 without affecting pGL3-KR1activity. A catalytically inactive form of CD2-P110α (R1130P), on the other hand, prevented the dexamethasone-induced activation of SGK1, but did not inhibit the activation of pGL3-KR1. However, expression of SGK1-S422D or CD2-P110α enhanced the transcriptional responses to maximally effective concentrations of dexamethasone and this effect occurred with no change in EC_{50}. Dexamethasone-induced (0.3–300 nM) activation of pGL3-KR1 was unaffected by inhibitors of PI3K (PI-103 and wortmanin) and by rapamycin, a selective inhibitor of the TORC1 (target of rapamycin complex 1) signalling complex. Dexamethasone-induced activation of the α-ENaC gene promoter can thus occur independently of SGK1/PI3K, although this pathway does provide a mechanism that allows this transcriptional response to dexamethasone to be enhanced.

Key words: airway epithelium, epithelial Na^+ channel (ENaC) dexamethasone, phosphoinositide 3-kinase (PI3K), reporter gene, serum- and glucocorticoid-induced protein kinase 1 (SGK1).

INTRODUCTION

The integrated functioning of the respiratory tract is dependent upon the controlled absorption of Na^+ from the liquid film that covers the lung/airway epithelia, and glucocorticoid hormones are important to the induction and maintenance of this Na^+-absorbing phenotype [1–3]. Epithelial Na^+ absorption occurs via a 'leak–pump' mechanism [4], in which the overall rate of Na^+ absorption is restricted by the rate of apical Na^+ entry, and this rate-limiting influx of Na^+ occurs via ENaCs (epithelial Na^+ channels), transport proteins composed of three subunits (α-, β- and γ-ENaC) encoded by separate genes [5,6]. In unstimulated cells, apical Na^+ permeability appears to be restricted by the continual internalization of ENaC, and this process is mediated by Nedd-4/2 (neural precursor cell expressed, developmentally down-regulated protein 4-2), an ubiquitin ligase that binds to WW domains (protein–protein interaction modules characterized by two conserved proline residues spaced 20–22 amino acids apart) on β- and γ-ENaC, thus targeting the ENaC channel complex for ubiquitination, internalization and degradation [7,8]. Glucocorticoids induce expression of SGK1 (serum- and glucocorticoid-induced protein kinase 1) [9,10], a regulatory kinase that phosphorylates Nedd-4/2, thus blocking this protein's interaction with ENaC. Activating SGK1 therefore allows ENaC to remain in the apical membrane, leading to a rise in Na^+ permeability and a stimulation of Na^+ absorption [7,8]. However, glucocorticoid hormones also control α-ENaC transcription by activating a GRE (glucocorticoid receptor response element) in this gene's promoter region, and this provides another mechanism that allows these hormones to contribute to the control of Na^+ absorption [11–13]. It is therefore interesting that studies of renal epithelia have indicated that SGK1 may also play a role in the control of α-ENaC transcription [14]. Since this may have implications for our understanding of the ways in which glucocorticoids control pulmonary Na^+ transport, the present study explores the relationship between cellular SGK1 activity and α-ENaC transcription in glucocorticoid-stimulated human airway epithelial cells.

EXPERIMENTAL

Cell culture and molecular biology

Experiments were undertaken using a human distal airway epithelial cell line (H441) that expressed an endogenous Na^+ conductance essentially identical with that associated with α-, β- and γ-ENaC co-expression [6]. Since the activity of this Na^+ conductance is strictly dependent upon glucocorticoid stimulation [15], these cells provide a valuable experimental system with which to explore the factors that allow these hormones to control Na^+ transport [15]. Standard techniques were used to maintain these cells in serial culture [16], and experiments were undertaken

Abbreviations used: Af9, ALL-1 fused gene from chromosome 9; CD2, cluster of differentiation 2; df, degrees of freedom; Dot1a, disruptor of telomeric silencing alternative splice variant a; ENaC, epithelial Na^+ channel; GRE, glucocorticoid response element; Nedd-4/2, neural precursor cell expressed, developmentally down-regulated protein 4-2; NDRG1, *N*-myc downstream regulated gene 1; PIM1 and 3, provirus integration site for Moloney murine leukaemia 1 and 3; SGK1, serum- and glucococorticoid-induced protein kinase; PI3K, phosphoinositide 3-kinase; TORC, target of rapamycin complex.

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using cells removed from culture flasks using trypsin/EDTA and plated on to 24-well (reporter gene assays, 7.5×10^4 cells/well) or six-well (Western analysis, 3×10^5 cells/well) plates. Cellular SGK1 activity was artificially increased by transfecting cells with a cDNA construct encoding a glutathione transferase-fusion protein incorporating a truncated form of SGK1 which lacked 60 N-terminal amino acid residues and which had been further modified by mutating SGK1-Ser^{422} to aspartate (SGK1-S422D). The N-terminal truncation increases the expression of this protein by preventing its degradation, and the S422D mutation allows SGK1 to be activated by PDK1 (3-phosphoinositide-dependent protein kinase 1) [10,18]. Taken together, these mutations confer a constitutively active phenotype [18]. Non-specific effects of the transfection and/or heterologous protein expression were controlled for using an analogous construct encoding a catalytically inactive form of SGK1, SGK1-K127A [18]. The role of PI3K (phosphoinositide 3-kinase) was explored by expressing a chimaeric protein consisting of the catalytic P110α domain of PI3K (PI3K-P110α) attached to the extracellular and transmembrane domains of the rat CD2 surface antigen. The CD2 domain effectively anchors the PI3K-P110α subunit to the inner surface of the membrane. PI3K activity was enhanced using a construct encoding wild-type PI3K-P110α (CD2-P110α), and the corresponding control construct (CD2-P110α-R1130P) incorporated a catalytically inactive mutant. This system has been detailed previously in [19].

Transcriptional activity of the α-ENaC promoter

Activation of the promoter region of the α-ENaC gene was assayed by transfecting cells on 24-well plates using Lipofectamine™ 2000 with a luciferase-linked reporter gene construct (pGL3-KR1) incorporating 2.2 kb of the α-ENaC gene sequence corresponding to nucleotides -1388 to $+830$ relative to the start site for the α-ENaC-1 transcript. This gene sequence includes exon 1A, intron 1, the start site for the α-ENaC-2 transcript and the GRE at position -141 to -155 that is known to regulate transcription [12,13]. In all experiments the transfected cells were initially maintained (24 h) in a fully defined culture medium prepared using dialysed serum devoid of glucocorticoids [16]. Glucocorticoid-deprived cells were exposed to this medium throughout the entire experimental period, whereas dexamethasone-stimulated cells were exposed to this synthetic glucocorticoid as detailed below. Experiments were terminated by lysing the cells so that luciferase formation could be quantified (Wallac 1420 Victor plate reading luminometer/Promega luciferase assay system) and normalized to the amount of cellular protein in each well, which was determined using Bradford reagent. Some experiments were undertaken using a reporter construct that had been modified by deleting (Stratagene QuikChange II site-directed mutagenesis kit) five nucleotides (position -150 to -155) in order to disrupt the GRE that has previously been documented in the α-ENaC gene promoter [12,13].

Experimental design and data analysis

All pGL3-KR1 activities were expressed as fold increases over the basal activity measured in cells expressing the empty pGL3 vector (pGL3-basic). Hormone-sensitive pGL3-KR1 activity was quantified by subtracting the activity measured in glucocorticoid-deprived cells from the activity measured in dexamethasone-stimulated cells, and all such data are shown as percentages of the activity measured in cells exposed to a maximally effective concentration of dexamethasone. To allow comparison between different data sets, the response to this standard stimulus was quantified in every experiment. Plots showing the relationship between hormone-sensitive pGL3-KR1 activity and dexamethasone concentration were constructed and sigmoid curves fitted to the pooled data by least-squares regression. Since the background activity was defined experimentally, the algorithm used to fit this curve (Grafit 5; Erithacus Software) estimated (i) the concentration of dexamethasone needed for half-maximal activation (EC_{50}); (ii) the maximal response (R_{max}), and (iii) the Hill Coefficient. This software package also provided an estimate of the standard error associated with each parameter and the statistical significance of any apparent differences between data sets could thus be tested by calculating a value of Student's t using the equation:

$$t = (Param_1 - Param_2)/\sqrt{(SE_1^2 - SE_2^2)}$$

where $Param_1$ and $Param_2$ are the respective parameters measured under different conditions (i.e. EC_{50} or R_{max}) and SE_1 and SE_2 represent their respective standard errors. Each curve was defined by six or seven different dexamethasone concentrations and was therefore fitted with three or four df (degrees of freedom). This, in turn, implies that the values of t derived from this equation are associated with six or eight df, and these were therefore used to estimate the two-tailed probabilities (P) that the two parameters are derived from the same statistical populations. Values of $P < 0.05$ were considered to be significant. Values of n denote the number of times a protocol was repeated using cells at different passage number and all results are means $\pm$ S.E.M.

Assay of SGK1 activity

Changes in cellular SGK1 activity were monitored using phospho-specific antibodies to monitor by Western analysis the phosphorylation of residues ($Thr^{346/356/366}$) within an endogenous protein (NDRG1; N-myc-downstream gene 1) that are phosphorylated by SGK1 but not by other kinases, including the closely related PKB (protein kinase B) [20–22]. Although it is now clear that changes to the phosphorylation status of these residues can provide a read-out of cellular SGK1 activity (see e.g. [22,23]), our experience is that there can be variations in the magnitudes of the responses measured in different experiments. To ensure that this effect did not confound analysis of the present data, all experiments were undertaken using strictly paired experimental designs in which the control and experimental cells were age-matched and at identical passage. Great care was taken to handle all protein samples identically and, in all experiments, proteins extracted from control and experimental cells were processed in parallel using identical reagents and then fractionated on the same gels so that they could be analysed in exactly the same way. A full account of this method [22], and a detailed review of its rationale [20,21], are published elsewhere.

RESULTS

Glucocorticoid-induced activation of the α-ENaC gene promoter

When expressed in glucocorticoid-deprived cells, the activity of the pGL3-KR1 reporter gene was 7.3 ± 1.0-fold greater ($n = 49$, $P < 0.0001$, Student's paired t test) than the activity measured in cells expressing the empty pGL3 vector. Dexamethasone (0.1 μM) consistently evoked luciferase synthesis in pGL3-KR1-expressing cells and, since this response was not seen in cells expressing the empty vector (results not shown), this finding confirms that this synthetic glucocorticoid normally activates the

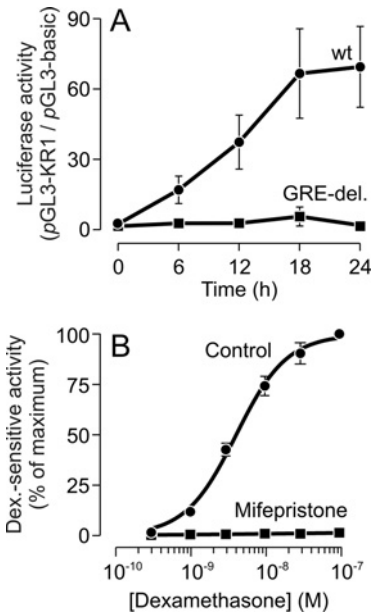

Figure 1 Dexamethasone-induced activation of pGL3-KR1

(**A**) Time courses showing the effects of dexamethasone (0.1 μM) upon luciferase accumulation in cells expressing either wild-type (wt) α-ENaC reporter construct (pGL3-KR1, $n = 10$) or the construct incorporating the mutant α-ENaC sequence lacking the functional GRE (GRE-del, $n = 5$). (**B**) Concentration–effect curves showing the effects of dexamethasone (18 h) upon the activity of pGL3-KR1 activity both under control conditions ($n = 8$) and in the presence of 2.5 μM mifepristone ($n = 8$).

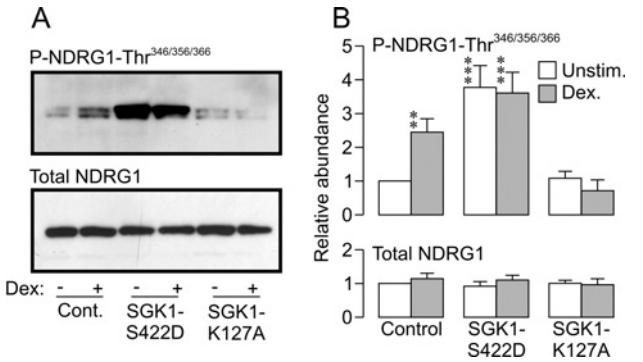

Figure 2 Activation of SGK1

(**A**) Control (i.e. cells transfected with empty vector; Cont.) cells and cells transiently expressing either SGK1-S422D or SGK1-K127A were maintained in hormone-free medium or stimulated with 0.1 μM dexamethasone (Dex) for 18 h. All cells were then lysed and 15 μg aliquots of cellular protein were fractionated by SDS/PAGE, blotted on to Hibond membranes which were then probed using antibodies against the Thr$^{346/356/366}$ phosphorylated form of NDRG1 (upper panel) or total NDRG1 (lower panel). (**B**) Densitometric analysis showing the pooled (means $\pm$ S.E.M.) from six independent experiments. Unstim., unstimulated; Dex., dexamethasone.

α-ENaC gene promoter. This response became apparent after $\sim$6 h, reached a plateau at $\sim$18 h (Figure 1A) and was abolished by disrupting the GRE that has previously been shown to regulate the activity of this gene promoter (Figure 1A). Experiments in which luciferase formation was quantified in cells exposed to 0.3–300 nM dexamethasone for 18 h showed that this response was concentration-dependent, and the EC_{50} was estimated to be 3.7 ± 0.4 nM (Figure 1B), a value similar to that reported in a previous study [25]. Concentrations of dexamethasone > 30 nM usually evoked maximal responses, although, in some experiments, high concentrations of dexamethasone evoked sub-maximal responses, indicating that this response can be subject to desensitization. The activation of this reporter gene was abolished by mifepristone, a glucocorticoid receptor antagonist (Figure 1B), and it is therefore clear that this response reflects glucocorticoid receptor-mediated activation of the GRE that has been previously described in the promoter region of the α-ENaC gene [11–13].

SGK1-induced phosphorylation NDRG1-Thr$^{346/356/366}$

Western blot analyses of protein extracted from unstimulated and dexamethasone-treated (0.1 μM, 18 h) cells expressing the empty pGL3 vector showed that this synthetic glucocorticoid increased the abundance of the Thr$^{346/356/366}$-phosphorylated NDRG1 with no effect upon the overall expression of this protein (Figure 2). This response was essentially identical with that reported in previous studies of untransfected cells [22] and it is therefore clear that exposure to transfection reagents does not alter this response. Parallel studies of glucocorticoid-deprived cells expressing SGK1-S422D revealed a clear stimulation of NDRG1-Thr$^{346/356/366}$ phosphorylation and this response, in common with the response to dexamethasone, occurred with no change in the overall NDRG1 abundance (Figure 2). Dexamethasone stimulation had no further effect upon the phosphorylation of NDRG1 in SGK1-S422D-expressing cells (Figure 2). Transient expression of SGK1-K127A had no effect upon the phosphorylation status of NDRG1-Thr$^{346/356/366}$ in dexamethasone-deprived cells, but this catalytically-inactive form of SGK1 suppressed the dexamethasone-induced phosphorylation of Thr$^{346/356/366}$ in NDRG1 (Figure 2). Since NDRG1-Thr$^{346/356/366}$ residues are phosphorylated by SGK1 [20], these results (see also [22]) show that this catalytically inactive form of SGK1 suppresses the hormonal activation of this kinase.

SGK1-induced activation of pGL3-KR1

To explore the role of SGK1 in the transcriptional response to dexamethasone, we assayed the transcriptional activity of pGL3-KR1 in control cells (i.e. cells expressing the empty vector) and in cells transiently expressing SGK1-S422D and SGK1-K127A. Analysis of data derived from glucocorticoid-deprived cells showed that the basal activity of pGL3-KR1 was $\sim$9-fold greater than the activity associated with the empty pGL3 vector (Figure 3A). SGK1-S422A-expressing cells displayed an essentially identical level of activity (Figure 3A) and, although the basal activity measured in cells expressing SGK1-K127A appeared slightly lower than control (Figure 3A), this effect was not statistically significant (one-way ANOVA). The data in Figure 3(B) show that dexamethasone normally caused concentration-dependent activation of pGL3-KR1 in SGK1-K127A-expressing cells, and the EC_{50} measured under these conditions (4.7 ± 0.5 nM), did not differ significantly from that measured in age-matched control cells at identical passage (Figure 3B). The maximal response to dexamethasone (R_{max}) was also essentially identical (93.6 ± 2.8 %) with the control, and so the expression of this inactive SGK1 mutant has no discernible effect upon the transcriptional response to dexamethasone. Analysis of the data derived from SGK1-S422D-expressing cells showed that this constitutively active mutant had no effect upon the responses to low concentrations of dexamethasone, but enhanced the responses to the highest concentrations tested (Figure 3B). The value of R_{max} measured in these cells (188 ± 13 %) was therefore greater ($t = 7.28$, df $= 8$, $P < 0.0001$) than the value measured in SGK1-K127A-expressing cells, and this effect occurred with no change in EC_{50} (5.9 ± 1.6 nM).

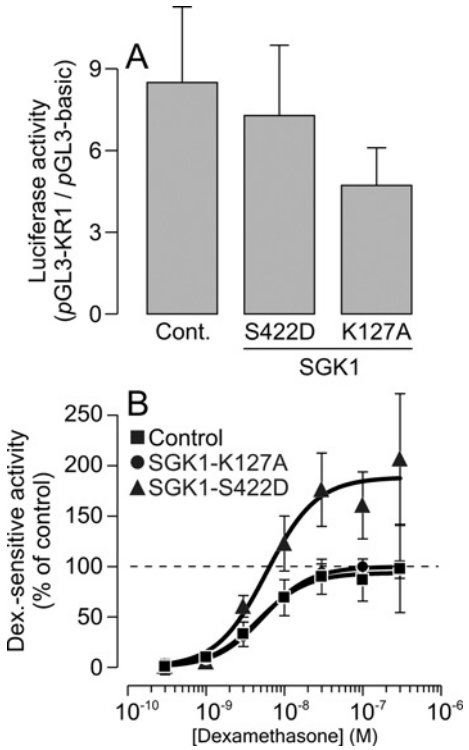

Figure 3 Role of SGK1 in α-ENaC transcription

(**A**) Luciferase formation (18 h, $n = 9$) was quantified in hormone-deprived cells co-expressing the α-ENaC reporter gene in conjunction with SGK1-S422D or SGK1-K127A; control (Cont.) cells expressed this reporter gene construct together with the empty pEGB vector. (**B**) Dexamethasone-induced (18 h) activation of pGL3-KR1 in control cells (i.e. cells expressing pGL3-KR1 and pEGB) and in cells co-expressing either SGK1-S442D or SGK1-K127A ($n = 8$). The continuous curves were fitted to the experimental data by least-squares regression. All results are normalized to the luciferase formation measured in cells expressing the empty pGL3 vector and are shown as means $\pm$ S.E.M.

PI3K-induced NDRG1-Thr$^{346/356/366}$ phosphorylation

Figure 4 shows the results of experiments that quantified NDRG1-Thr$^{346/356/366}$ phosphorylation in glucocorticoid-deprived and dexamethasone-stimulated cells transiently expressing the chimaeric proteins incorporating the catalytic PI3K-P110α subunit. Results derived from control cells confirmed (in the present study and [22]) that dexamethasone (0.1 μM, 18 h) evokes the phosphorylation of these residues with no effect upon the overall NDRG1 abundance, confirming that glucocorticoids normally increase SGK1 activity (see [20,22]). Transient expression of CD2-P110α also evoked NDRG1-Thr$^{346/356/366}$ phosphorylation with no effect upon the overall expression, indicating that artificially increasing cellular PI3K activity mimics the effects of glucocorticoid stimulation by activating endogenous SGK1 (Figure 4). Dexamethasone stimulation had no further effect upon the phosphorylation of NDRG1-Thr$^{346/356/366}$ in CD2-P110α-expressing cells (Figure 4). Expression of CD2-P110α-R1130, which incorporates a catalytically inactive form of the PI3K-P110α subunit, had no effect upon NDRG1 Thr$^{346/356/366}$ phosphorylation in glucocorticoid-deprived cells, but prevented the increased phosphorylation of these residues that was normally seen in glucocorticoid-stimulated cells (Figure 4). Since PI3K is known to control the phosphorylation/activation of SGK1 [18,26], the results from the present study indicate that the transient expression of CD2-P110α-R1130 exerts a dominant-negative effect by suppressing the glucocorticoid-induced activation of SGK1 (Figure 4).

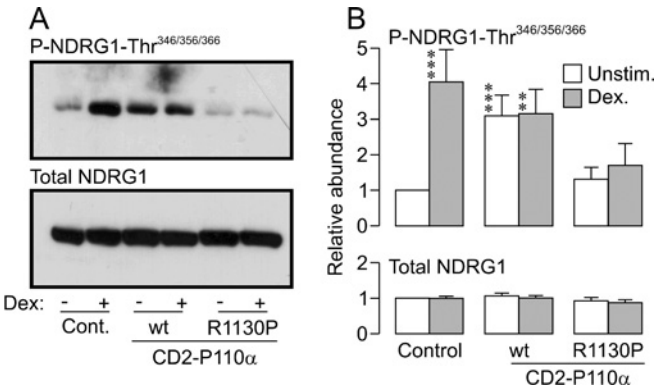

Figure 4 Effects of increasing cellular PI3K activity

(**A**) Control cells (i.e. cells transfected with empty vector; Cont.) and cells transiently expressing either CD2-P110α or CD2-P110α-R1130P were either maintained in hormone-free medium or stimulated with 0.1 μM dexamethasone (Dex) for 18 h. All cells were then lysed and 15 μg aliquots of cellular protein fractionated so that the cellular abundance of Thr$^{346/356/366}$-phosphorylated NDRG1 (upper panel) and total NDRG1 (lower panel) could be assayed by Western analysis. (**B**) Densitometric analysis showing the pooled means $\pm$ S.E.M. from ten independent experiments. Unstim., unstimulated; Dex., dexamethasone; wt, wild-type.

PI3K-induced activation of pGL3-KR1

Experiments in which the activity of the α-ENaC reporter gene construct was monitored in hormone-deprived cells confirmed (in the present study and [25]) that the basal activity of this construct is $\sim$10-fold greater than that associated with the empty vector (Figure 5A). Initial examination of the results of experiments in which transcriptional activity was quantified in cells expressing CD2-P110α or CD2-P110α-R1130P suggested that the expression of these chimaeric proteins might inhibit pGL3-KR1 (Figure 5A). However, these apparent effects did not reach statistical significance (one-way ANOVA), and so neither construct has any direct effect upon the transcriptional activity of the promoter region of the α-ENaC gene. These experiments also explored the effects of dexamethasone (18 h, 1–300 nM) upon pGL3-KR1 activity in cells expressing these chimaeric proteins. As anticipated, studies of control cells confirmed that this hormone normally induces concentration-dependent activation of the α-ENaC gene promoter (see above). Although this response was also seen in CD2-P110α-R1130P-expressing cells, analysis of these results indicated that the maximal response to dexamethasone ($R_{max} = 1.84 \pm 0.06$, Figure 5B) was greater than control ($P < 0.05$). However, the most important result to emerge from these studies was that the value of R_{max} measured in cells expressing the catalytically active CD2-P110α protein was greater ($t = 8.11$, df $= 4$, $P < 0.0001$) than the maximal response measured in cells expressing CD2-P110α-R1130P. It is therefore clear that increasing cellular PI3K activity enhances the dexamethasone-induced activation of the α-ENaC reporter gene construct.

Effects of cell stress

As well as being induced by glucocorticoid hormones, data from several different cell types show that SGK1 activity/expression is enhanced by stressful stimuli [9,27–31], and we therefore explored the effects of such stimuli on SGK1 activity in H441 cells. Initial studies ($n = 5$) showed that 2 h exposure to a

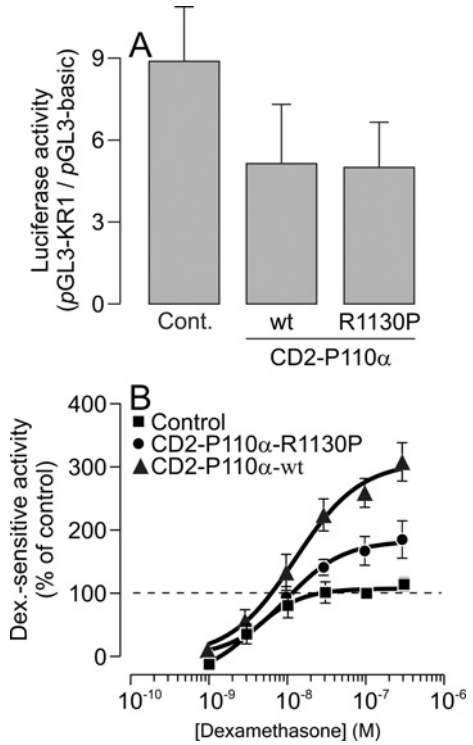

Figure 5 Role of PI3K in α-ENaC transcription

(**A**) The experimental protocol shown in Figure 3 was used to monitor the transcriptional activity of pGL3-KR1 in hormone-deprived control (Cont.) cells and in expressing either CD2-P110α (wild-type, wt) or CD2-P110α-R1130P. (**B**) Dexamethasone-induced (18 h) activation of pGL3-KR1 in control cells and in cells expressing either CD2-P110α-wt or CD2-P110α-R1130P ($n = 9$). The continuous curves were fitted to the experimental data by least-squares regression. All data are normalized to the luciferase formation measured in cells expressing the empty pGL3 vector and are shown as means $\pm$ S.E.M.

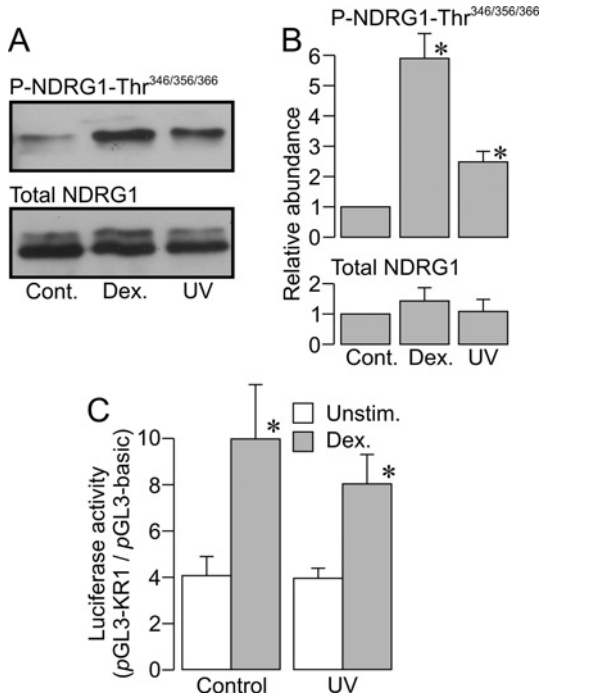

Figure 6 Effects of UV irradiation upon the phosphorylation of NDRG1-Thr$^{346/356/366}$ and the activity of the α-ENaC reporter gene construct

(**A**) Western blots showing the effects of dexamethasone (Dex., 0.1 μM, 6 h) and UV irradiation upon the cellular abundance of Thr$^{346/356/366}$-phosphorylated and total NDRG1. Cont., control. (**B**) Densitometric analysis showing the pooled means $\pm$ S.E.M. from five independent experiments; asterisks denote statistically significant differences from the control (Cont.) value (Student's paired t test). Dex., dexamethasone (**C**) Results of a series of experiments ($n = 5$) undertaken using a strictly paired experimental design that explored the effects of UV irrdiation upon the dexamethasone (Dex.) induced (0.1 μM, 6 h) activation of the α-ENaC reporter gene construct. Asterisks denote statistically significant effects of dexamethasone (Student's paired t test). Unstim., unstimulated.

hyperosmotic external solution (prepared by adding 300 mM sorbitol to the standard culture medium) had no effect upon NDRG1 phosphorylation assayed after a 12 h recovery period. Similarly exposing cells ($n = 4$) to increased ambient temperature (42 °C, 1 h) or H_2O_2 (0.5 mM, 1–2 h) also had no effect upon the phosphorylation of these residues. However, exposing cells to a brief <1 s pulse of UV light (50 J $\cdot$ m^2; Spectronic Corporation Spectrolinker) consistently increased the phosphorylation of NDRG1-Thr$^{346/356/366}$ assayed after a 2 h recovery period, and this stimulus therefore provides a way of activating endogenous SGK1 independently of stimulating hormones (Figures 6A and 6B). However, subsequent experiments showed that irradiating the cells with UV light in this way had no direct effect upon the transcriptional activity of the α-ENaC gene promoter and also failed to modify the transcriptional response to dexamethasone (Figure 6C).

Effects of PI3K inhibitors

Figure 7 shows the results of experiments that used a strictly paired protocol to study the effects of PI3K inhibitors upon the dexamethasone-induced (0.3–100 nM) activation of pGL3-KR1. Although LY294002 (50 μM) had no effect upon the EC_{50} for dexamethasone (control: 3.4 ± 0.4 nM; LY294002-treated: 3.2 ± 0.5 nM), this substance caused $\sim$30 % inhibition ($t = 13.41$, df $= 6$, $P < 0.0001$) of the maximal response to this hormone (Figure 7A). Wortmannin, on the other hand, had no effect upon EC_{50} (control: 4.1 ± 0.3 nM; wortmannin-treated: 6.4 ± 3.8 nM) or the maximal response (Figure 7B). Although PI-103 appeared to cause a slight leftward shift in the concentration–response curve, this effect was not statistically significant and so this compound did not alter the EC_{50} (control: 3.7 ± 0.09 nM; PI-103-treated: 2.1 ± 0.1 nM). PI-103 also had no effect upon the magnitude of the maximal response (Figure 7C). Although LY294002, wortmannin and PI-103 are often used as selective inhibitors of PI3K, these compounds also inhibit TORC1, a signalling complex dependent upon mTOR (mammalian target of rapamycin) that controls many aspects of cellular physiology. We therefore also explored the effects rapamycin, an exquisitely selective inhibitor of TORC1 [32]. Rapamycin had no effect upon the EC_{50} for dexamethasone (control: 3.2 ± 0.3 nM; rapamycin-treated: 3.2 ± 0.5 nM), but enhanced the maximal response to this hormone ($t = 7.13$, df $= 6$, $P = 0.0004$) by $\sim$75 % (Figure 7D).

Analysis of protein extracted from glucocorticoid-deprived cells that had been maintained under control conditions or exposed to wortmannin/PI-103 showed that both substances reduced ($\sim$70 %) the abundance of Thr$^{346/356/366}$-phosphorylated NDRG1, with no effect upon the overall levels of NDRG1. As anticipated, dexamethasone (0.1 μM) normally increased the phosphorylation of NDRG1-Thr$^{346/356/366}$ (Figure 8) and, although there was some evidence of a residual response in the wortmannin/PI-103-treated cells, this effect did not reach statistical significance (Figure 8). These experiments also explored the effects of LY294002 and, as anticipated by our previous work [22], this compound caused essentially complete dephosphorylation of NDRG1 and totally

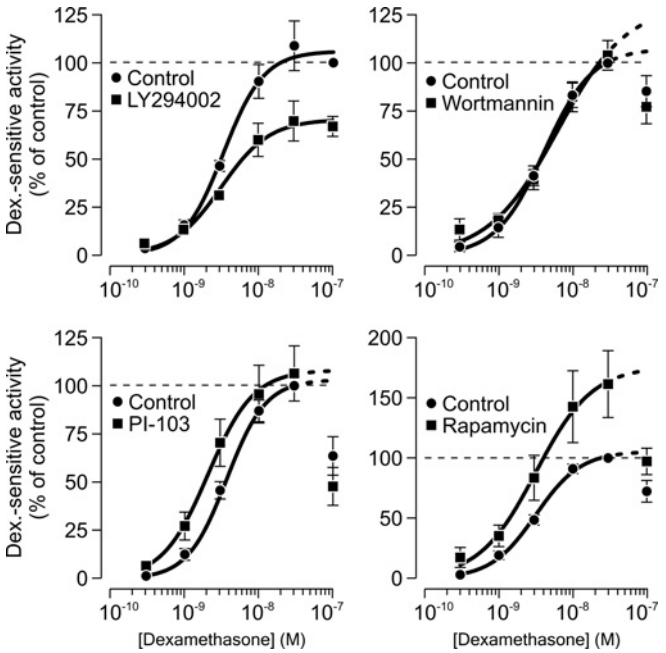

Figure 7 Dexamethasone-induced activation of the α-ENaC gene promoter in cells treated with PI3K inhibitors or rapamycin

All experiments were undertaken using a strictly paired protocol in which dexamethasone-induced (0.3–100 nM, 18 h) activation of pGL3-KR1 was monitored under control conditions and in the presence of either 50 μM LY294002 (**A**, $n=13$); 0.1 μM wortmannin (**B**, $n=8$); 0.5 μM PI-103 (**C**, $n=8$), or 100 nM rapamycin (**D**, $n=12$). All results were normalized to the control response evoked by a maximally effective concentration of dexamethasone. This usually occurred in response to 100 μM, but in some experiments this high concentration of hormone evoked a submaximal response (as shown in **B**, **C** and **D**) and in these experiments results were normalized to the response evoked by 30 nM dexamethasone. Results are shown as means $\pm$ S.E.M. and sigmoid curves were fitted to the pooled data by least-squares regression.

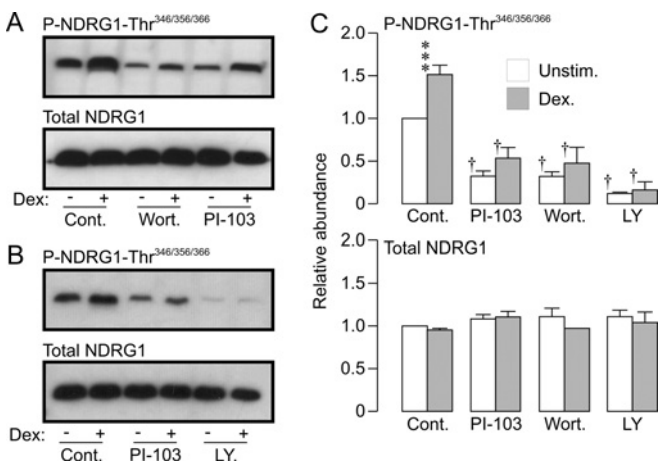

Figure 8 Effects of PI3K inhibitors on the phosphorylation of NDRG1-Thr$^{346/356/366}$

(**A**) Western blots showing the effects of wortmannin (Wort.) (0.1 μM) and PI-103 (0.5 μM) upon the cellular abundance of Thr$^{346/356/366}$-phosphorylated and total NDRG1 in glucocorticoid-deprived and dexamethasone (Dex)-stimulated (0.1 μM) cells. This protocol was repeated five times using cells at different passage number. Cont. control (**B**) Results from a directly analogous experiment showing effects of PI-103 (0.5 μM) and LY294002 (Ly; 50 μM). This protocol was repeated five times. Dex, dexamethasone; Cont., control. (**C**) Densitometric analysis showing the pooled means $\pm$ S.E.M. from all experiments. Unstim., unstimulated; Dex, dexamethasone; Cont., control; Wort., Wortmannin; LY, LY294002.

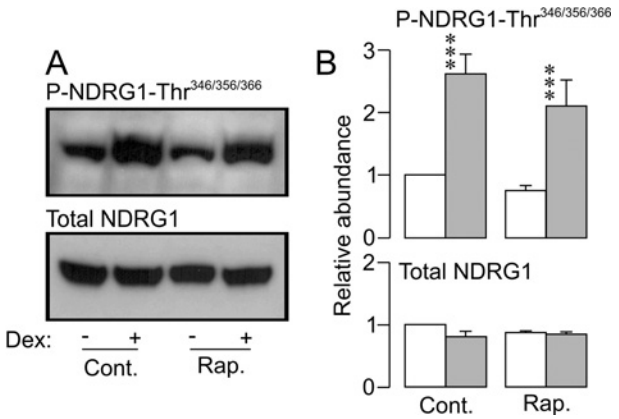

Figure 9 Effects of rapamycin upon the phosphorylation of NDRG1-Thr$^{346/356/366}$

(**A**) Western blots showing the effects of rapamycin (0.1 μM) on the cellular abundance of Thr$^{346/356/366}$-phosphorylated and total NDRG1 in glucocorticoid-deprived and dexamethasone (Dex)-stimulated (0.1 μM) cells. (**B**) Densitometric analysis showing the pooled means $\pm$ S.E.M. from five independent experiments. Rap., rapamycin.

abolished the response to dexamethasone. Further experiments showed that rapamycin had no significant effect upon the phosphorylation status of these residues in either glucocorticoid-deprived or dexamethasone-stimulated cells (Figure 9).

DISCUSSION

Dexamethasone-induced activation of the α-ENaC gene promoter

When expressed in glucocorticoid-deprived cells, the α-ENaC reporter gene construct displayed a level of activity 5–10-fold greater than that associated with the empty vector, and such low basal activity has been documented in previous studies of the rat and human α-ENaC promoters [11–13,25,33,34]. Dexamethasone consistently activated this reporter gene construct via a mechanism dependent upon the GRE in the α-ENaC promoter [11–13,25,33,34], and the fact that this response was abolished by mifepristone, a glucocorticoid receptor antagonist, provides further evidence for the involvement of these receptors. The response to dexamethasone was concentration-dependent and the half-maximal activation occurred at $\sim$4 nM (see also [25]). It is therefore interesting that the oral administration of 1 mg of dexamethasone increases the circulating concentration to $\sim$7 nM [35], whereas the intravenous administration of 5 mg leads to a circulating concentration of 50–100 nM [36,37]. These clinically relevant doses of dexamethasone therefore produce circulating concentrations sufficient to influence α-ENaC transcription and this response may therefore contribute to the clinical effects of this synthetic glucocorticoid.

Dexamethasone-induced phosphorylation of NDRG1-Thr$^{346/356/366}$

Stimulation with dexamethasone also evoked phosphorylation of NDRG1-Thr$^{346/356/366}$ and, since these residues are phosphorylated by SGK1 but not by other related kinases [20,21], this confirms that glucocorticoids activate SGK1 in these cells [10]. Our recently published data [22] show that this response peaks after $\sim$2 h, and the fact that elevated SGK1 activity is still evident after 18 h shows that the dexamethasone-induced activation of the α-ENaC promoter coincides with increased SGK1 activity, an observation consistent with the hypothesis that SGK1 is involved in this transcriptional response [14,38].

Effects of SGK1-S422D/SGK1-K127A

Expressing SGK1-S422D in glucocortcoid-deprived cells evoked NDRG1-Thr$^{346/356/366}$ phosphorylation, and it is therefore clear that the expression of this constitutively active protein provides a way of increasing cellular SGK1 activity independently of stimulating hormones (see also [22]). However, despite this clear finding, expressing SGK1-S422D in glucocorticoid-deprived cells did not alter the activity of the α-ENaC gene promoter and it is therefore clear that a substantial increase in cellular SGK1 activity does not provide a stimulus sufficient to mimic the transcriptional response to dexamethasone. This was surprising, since previous studies of renal epithelia had indicated that SGK1 was important to the control of α-ENaC gene transcription [38]. However, our studies of dexamethasone-stimulated cells showed that SGK1-S422D expression enhanced the response to concentrations of dexamethasone > 10 nM, and it is therefore clear that artificially imposed increases in cellular SGK1 activity can enhance the glucocorticoid-induced activation of the α-ENaC gene promoter under certain experimental conditions.

Parallel studies of glucocorticoid-deprived cells expressing SGK1-K127 showed that this mutant protein had no effect upon NDRG1-Thr$^{346/356/366}$ phosphorylation, and it is therefore clear that this catalytically inactive protein does not alter cellular SGK1 activity. The activation of SGK1 seen in cells expressing SGK1-S422D cannot, therefore, be attributed to increased activity of endogenous SGK1 evoked by exposure to transfection reagents and/or to the expression of heterologous protein. However, the expression of SGK1-K127A did block the dexamethasone-induced increase in NDRG1-Thr$^{346/356/366}$ phosphorylation, and this catalytically inactive SGK1 mutant therefore seems to display a dominant-negative phenotype. Transient expression of this mutant protein therefore provides a way of disrupting the hormonal activation of endogenous SGK1 but, despite this clear finding, SGK1-K127A expression had no effect upon the transcriptional response to dexamethasone. Although increases in cellular SGK1 activity can augment the transcriptional response to dexamethasone (see above), it therefore appears that the hormonal activation of endogenous SGK1 is not necessary for the glucocorticoid-induced activation of the α-ENaC gene promoter.

Effects of CD2-P110α/CD2-P110α-R1130P

Three isoforms of SGK have been identified in mammalian cells (SGK1–3) [27,39,40] and, although these share a high degree of homology in the catalytic domain, they differ in N-terminal structure. This variability is significant, since this region of SGK1 influences protein stability and cellular localization [18,41–43]. Moreover, although SGK1 is the only isoform that is hormone-sensitive, three splice variants of human SGK1 have been identified and these mRNA species encode proteins that also display different N-terminal sequences [44]. These SGK1 variants may therefore display different patterns of degradation and different cellular locations, and this heterogeneity may explain, at least in part, the diverse cellular functions of this kinase [10]. The fact that 60 N-terminal residues were deleted from the constitutively active form of SGK1 used in the present study implies that this modified protein is not degraded as rapidly as full-length SGK1 [18]. Moreover, this N-terminal deletion may also cause the SGK1-S422D protein to be located to an inappropriate cellular compartment and so, although SGK1-S422D can clearly increase cellular SGK1 activity, the transient expression of SGK1-S422 may not reproduce the effects of activating endogenous SGK1.

We therefore sought to identify a way of activating endogenous SGK1 independently of stimulating hormones and, since the catalytic activity of SGK1 is dependent upon PI3K-regulated phosphorylation [9,18,23,26], we explored the effects of transiently expressing a chimaeric protein encoding a membrane-anchored form of the catalytic PI3K-P110α subunit [19]. Expressing this PI3K-activating protein in glucocorticoid-deprived cells clearly evoked NDRG1-Thr$^{346/356/366}$ phosphorylation, indicating increased activity of endogenous SGK1 [18,20,21,26]. Moreover, although a catalytically inactive control construct (CD2-P110α-R1130P) did not alter NDRG1 phosphorylation, this mutant protein did prevent the dexamethasone-induced increase in NDRG1-Thr$^{346/356/366}$ phosphorylation, indicating that this chimaeric protein displays a dominant–negative phenotype. It is therefore interesting that electrophysiological studies undertaken in this laboratory (M. Gallacher and S.M. Wilson, unpublished work) have shown that the expression of CD2-P110α-R1130P can partially suppress the glucocorticoid-induced Na^+ current in these cells [15,16]. Transient expression of CD2-P110α-R1130P thus seems to provide another way of blocking the glucocorticoid-induced activation of SGK1.

Expressing these chimaeric proteins in glucocorticoid-deprived cells had no effect upon the transcriptional activity of the α-ENaC gene promoter, indicating that PI3K-mediated activation of SGK1 does not mimic the transcriptional response to dexamethasone. Interestingly, although it has no catalytic activity, expressing CD2-P110α-R1130P did enhance the transcriptional response to dexamethasone. Although this unexpected response to a catalytically inactive protein cannot be mediated via PI3K or SGK1, it is important to remember that this mutant protein would almost certainly suppress the phosphorylation of PI3K targets other than SGK1. It is therefore possible that a PI3K-dependent signalling pathway may normally exert inhibitory control over α-ENaC gene transcription. However, the most important result to emerge from these experiments was that expression of CD2-P110α augmented the transcriptional response to dexamethasone to a level greater than that measured in CD2-P110α-R1130P-expressing cells. This result accords well with the data derived from cells expressing the modified forms of SGK1 and, taken together, these two sets of independent experiments indicate that artificially imposed increases in cellular SGK1 activity do not mimic the effects of glucocorticoid stimulation but do enhance the transcriptional responses to these hormones.

Pharmacological inhibition of PI3K

Studies of cells expressing SGK1-K127A or CD2-P110α-R1130P showed that glucocorticoids continue to activate the α-ENaC promoter even if the activation of endogenous SGK1 is prevented and so, although SGK1 augments the transcriptional response to dexamethasone (see above), increased SGK1 activity does not seem to be necessary for the hormonal activation of the α-ENaC gene promoter. Since this contrasts with the situation in renal epithelia where SGK1 is intimately involved in the control of α-ENaC transcription [14,38], further experiments explored the effects of pharmacological inhibitors of PI3K, since such compounds characteristically cause a profound loss of SGK1 activity (see e.g. [18,22,23]). Neither wortmanin nor PI-103 had any effect upon the transcriptional response to dexamethasone, and although LY294002 reduced the maximal response to this hormone, this effect was relatively modest ($\sim 30\,\%$ inhibition) and residual responses could clearly be seen in LY294002-treated cells. Parallel analyses of extracted protein showed that LY294002, PI-103 and wortmannin all reduced the phosphorylation of NDRG1-Thr$^{346/356/366}$, indicating these substances all cause substantial inactivation of endogenous SGK1 [20,21]. However, although LY294002 appeared to cause a complete

loss of activity (see also [22]), small amounts of $Thr^{346/356/366}$-phosphorylated NDRG1 could still be detected in PI-103-treated and wortmannin-treated cells, suggesting that these compounds may not inactivate SGK1 fully. This discrepancy was surprising, since all three compounds were used at concentrations thought to cause complete inhibition of PI3K in intact cells [32], and it may be relevant that, as well as inhibiting PI3K, LY294002 is known to inactivate a number of protein kinases including GSK3β (glycogen synthase kinase 3β), PLK3 (polo-like kinase 3), CK2, PIM1 (provirus integration site for Moloney murine leukaemia 1) and PIM3, and it has been therefore suggested that the use of LY294002 as a selective PI3K inhibitor should be discontinued [32]. However, even if we accept that LY294002 is the only tested compound that was able to cause complete inhibition of PI3K, then the present data show that essentially complete loss of endogenous SGK1 activity causes only a modest reduction of the transcriptional response to dexamethasone.

Effects of rapamycin

Although often referred to as PI3K inhibitors, LY294002, wortmannin and PI-103 also inhibit the TORC1 signalling complex [45,46], and this could be significant for the present study, since it has recently been proposed [47] that TORC1 catalyses the phosphorylation of SGK1-Ser^{422}, implying that TORC1 plays an important role in the control of cellular SGK1 activity (see [18,26]). We therefore also explored the effects of rapamycin, since this compound has been shown to act as an exquisitely selective inhibitor of TORC1 [32]. The first such studies showed that rapamycin augmented the glucocorticoid-induced activation of the α-ENaC gene promoter, and it is therefore clear that the inhibitory action of LY294002 described above cannot be attributed to an effect on TORC1. Indeed, these data also suggest that tonic activity of TORC1 may normally repress the activity of this gene promoter. However, the most important result to emerge from these studies was that rapamycin had no discernible effect upon NDRG1-$Thr^{346/356366}$ phosphorylation in glucocorticoid-deprived or dexamethasone-stimulated cells. Since separate experiments in this laboratory have shown that 2–3 h exposure to 0.1 μM rapamycin essentially abolishes TORC1 activity in H441 cells (assayed by monitoring the phosphorylation of S6K-Thr^{389}; G. Watt and S.C. Land, personal communication) this result, in contrast with the data presented by Hong et al. [47], indicates that TORC1 is not important to the control of SGK1. This finding does, however, accord well with results recently presented by García-Martínez and Alessi [23], who showed that the apparent phosphorylation of SGK1-Ser^{422} by TORC1 is almost certainly an artefact.

Significance of findings from the present study

Recently, studies of renal epithelial have indicated that α-ENaC transcription may be inhibited by a repressor complex [Dot1a (disruptor of telomeric silencing alternative splice variant a)/Af9 (ALL-1 fused gene from chromosome 9)] that associates with the α-ENaC gene promoter. This complex appears to be a substrate for SGK1, which phosphorylates Af9 at Ser^{435} thus allowing Dot1a to dissociate from this gene region and this, in turn, seems to permit unimpeded transcription [38]. It was therefore suggested that SGK1 is centrally important to the hormonal control of α-ENaC transcription [14,38], and this mechanism could explain the augmentation of the glucocorticoid-induced α-ENaC transcription that we observe in cells expressing SGK1-S422D or CD2-P110α. However, it is important to remember that transient expression of such constitutively active proteins would almost certainly cause hyperactivation of SGK1/PI3K, and it is therefore interesting that our results also show that dexamethasone can activate the α-ENaC gene promoter even when signalling via SGK1/PI3K has been blocked. Moreover, although irradiation with UV light increased the activity of endogenous SGK1 via a pathophysiological mechanism, this manoeuvre did not alter the activity of the α-ENaC reporter gene construct in unstimulated or dexamethasone-stimulated cells. Although it is abundantly clear that glucocorticoids do activate SGK1 in H441 cells (as shown in the present study and in [24]), the results from the present study, in contrast with those presented by Zhang et al. [38], suggest that this kinase can only play a very minor role in the hormonal control of α-ENaC transcription. It is therefore interesting that the quantification of mRNA levels presented by Zhang et al. (see Figure 6E in [38]) show that sgk1 gene deletion has only a modest ($\sim$30 % inhibition) effect upon α-ENaC mRNA abundance in the kidneys of mice fed a low-Na^+ diet. This result, in common with the results from the present study, indicates that substantial α-ENaC gene transcription must occur independently of SGK1 [38]. Moreover, in the lungs, α-ENaC gene deletion prevents the absorption of alveolar fluid that normally occurs during the perinatal period and thus causes death through severe respiratory distress within $\sim$48 h of birth [48]. Genetic deletion of the sgk1 gene, on the other hand, does not cause an overt pulmonary phenotype [49], indicating that at least some α-ENaC gene transcription must be able to occur independently of this kinase.

AUTHOR CONTRIBUTION

The experimental work was jointly undertaken by Niall McTavish and Jennet Getty. Ann Burchell and Stuart Wilson were primarily responsible for the design of the study and the initial drafting of the manuscript. All authors contributed to the study by joining in discussions of experimental data and by suggesting improvements to the design of individual experiments. All authors also contributed to the revision/editing of the manuscript and approved the final version.

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