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SGK1 activity in Na$^+$ absorbing airway epithelial cells monitored by assaying NDRG1-Thr$^{346/356/366}$ phosphorylation

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Abstract Studies of HeLa cells and serum- and glucocorticoid-regulated kinase 1 (SGK1) knockout mice identified threonine residues in the n-*myc* downstream-regulated gene 1 protein (NDRG1-Thr$^{346/356/366}$) that are phosphorylated by SGK1 but not by related kinases (Murray et al., Biochem J 385:1–12, 2005). We have, therefore, monitored the phosphorylation of NDRG1-Thr$^{346/356/366}$ in order to explore the changes in SGK1 activity associated with the induction and regulation of the glucocorticoid-dependent Na$^+$ conductance (G_{Na}) in human airway epithelial cells. Transient expression of active (SGK1-S422D) and inactive (SGK1-K127A) SGK1 mutants confirmed that activating SGK1 stimulates NDRG1-Thr$^{346/356/366}$ phosphorylation. Although G_{Na} is negligible in hormone-deprived cells, these cells displayed basal SGK1 activity that was sensitive to LY294002, an inhibitor of 3-phosphatidylinositol phosphate kinase (PI3K). Dexamethasone (0.2 μM) acutely activated SGK1 and the peak of this response (2–3 h) coincided with the induction of G_{Na}, and both responses were PI3K-dependent. While these data suggest that SGK1 might mediate the rise in G_{Na}, transient expression of the inactive SGK1-K127A mutant did not affect the hormonal induction of G_{Na} but did suppress the activation of SGK1. Dexamethasone-treated cells grown on permeable supports formed confluent epithelial sheets that generated short circuit current due to electrogenic Na$^+$ absorption. Forskolin and insulin both stimulated this current and the response to insulin, but not forskolin, was LY294002-sensitive and associated with the activation of SGK1. While these data suggest that SGK1 is involved in the control of G_{Na}, its role may be minor, which could explain why *sgk1* knockout has different effects upon different tissues.

Keywords Glucocorticoids · Epithelial Na$^+$ channel · n-*myc* downstream-regulated gene 1 · Cell signaling

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Introduction

Many responses to glucocorticoid and mineralocorticoid hormones involve the activation of serum- and glucocorticoid-regulated kinase 1 (SGK1) and genetic deletion of the *sgk1* gene prevents the aldosterone-mediated increase in renal Na$^+$ retention that normally occurs when dietary Na$^+$ is reduced [53]. However, in the distal colon, *sgk1*

knockout also leads to a paradoxical stimulation of Na$^+$ absorption and does not prevent the increased absorption of fecal Na$^+$ seen in Na$^+$-restricted animals [39]. It is also clear that *sgk1* deletion does not cause overt lung symptoms despite the fact that the integrated functioning of the respiratory tract is dependent upon glucocorticoid-induced absorption of Na$^+$ from the liquid film that covers the lung/airway epithelia [3, 9, 30] and so, although SGK1 does seem to be involved in the control of epithelial Na$^+$ absorption [see, e.g., 5, 6, 17, 26, 44], the way in which this kinase contributes to this regulated ion transport process is not fully understood [12, 19, 40, 49, 51].

SGK1 belongs to the AGC subfamily of protein kinases that also includes protein kinase B (PKB), ribosomal S6 kinases (RSKs, S6Ks), and the mitogen/stress-activated kinases. However, SGK1 most closely resembles PKB and these enzymes can phosphorylate the same proteins, especially if their activities are assayed in vitro or using cellular over expression systems [25, 34]. However, it is also clear that SGK1 and PKB selectively phosphorylate certain proteins in vivo [31] and the product of n-*myc* downstream gene 1 (NDRG1) has recently been identified as a physiological substrate for SGK1 but not PKB, RSK1, or S6K1 [31, 32]. This was initially surprising since the NDRG1 residues that are phosphorylated by SGK1 (Thr$^{346/356/366}$) form part of canonical AGC substrate motifs within a C-terminal nonapeptide (Arg-Ser-Arg-Ser-His-Thr-Ser-Glu-Gly) that is repeated three times, and this location predicts phosphorylation by other AGC kinases. However, studies of synthetic peptides identified residues within the nonapeptide that prevented phosphorylation by PKB and RSK1 [32] and NDRG1-Thr$^{346/356/366}$ phosphorylation in HeLa cells and mouse tissues is strictly dependent upon the expression of SGK1 [31]. Although SGK1 also phosphorylates other residues within this protein (Thr^{228} and Ser^{330}), antibodies that recognize the phosphorylation of these residues cross-react with the equivalent residues in the closely related NDRG2 protein (Thr^{330}, Ser^{332}). In contrast, the SGK1 consensus sequences that include Thr$^{346/356/366}$ are found within a repeated nonapeptide that is not found in NDRG2, NDRG3, or NDRG4. Antibodies against this nonapeptide thus show almost no cross-reactivity with other phosphoproteins [31].

Since the phosphorylation of NDRG1-Thr$^{346/356/366}$ can be assayed using well-characterized antibodies [31], these data effectively establish a new way of monitoring cellular SGK1 activity which could simplify physiological studies of this enzyme [31]. In the present study, we, therefore, use this new approach to monitor the changes in SGK1 activity associated with the induction and hormonal regulation of the glucocorticoid-dependent Na$^+$ conductance (G_{Na}) in a human airway epithelial cell line [11, 13].

Materials and methods

Cell culture

H441 cells were routinely cultured in RPMI 1640 medium supplemented with fetal bovine serum (FBS, 8.5%), newborn calf serum (NCS, 8.5%), glutamine (2 mM), transferrin (5 μg mL^{-1}), selenium (5 ng mL^{-1}), and an antibiotic/antimycotic mixture (Sigma Chemical, Poole, Dorset, England). For experiments, cells removed from culture flasks using trypsin/ethylenediaminetetraacetic acid (EDTA) were plated onto glass cover slips, 10 cm Petri dishes, or Costar Snapwell (Corning BV, Schipol-Rijk, The Netherlands) culture membranes [37]. After approximately 24 h incubation in standard medium, the cells were washed and incubated in medium prepared using FBS (8.5%) that had been dialyzed to remove hormones/growth factors. Hormone-deprived cells were maintained in this medium for 24–36 h before being used in experiments while hormone-stimulated cells were exposed to dexamethasone (0.2 μM) as described in the text.

Transient transfection

Cells were transfected (Lipofectamine Transfection Reagent, Invitrogen, UK) with *p*GL3 plasmids (Invitrogen; 1–1.5 μg per well) incorporating cDNA sequences encoding modified forms of SGK1. The construct used to enhance cellular SGK1 activity encoded a glutathione *S*-transferase (GST) fusion protein incorporating a truncated form of SGK1 lacking 60 N terminus amino acid residues that had been further modified by mutating Ser^{422} to Asp (SGK1-S422D). The N-terminal truncation induces a 20-fold to 250-fold increase in protein expression by preventing protein degradation while the S422D mutation allows this truncated protein to be more readily activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) [25, 26]. Taken together, these changes confer a constitutively active phenotype upon this mutant [25]. We also explored the effects of expressing a construct encoding an analogous GST/truncated SGK1 protein in which Lys^{127} had been mutated to Ala (SGK1-K127A). This disrupts the protein's ATP-binding site creating a catalytically inactive form of the enzyme [25]. Cells used for electrophysiological studies were cotransfected with a second plasmid (*p*EGFP, 0.1 μg) encoding green fluorescent protein (GFP) which allowed successfully transfected cells to be identified by GFP fluorescence. Membrane currents were recorded from these cells after approximately 24–26 h (see below). Nonspecific effects of the transfection procedure and/or expression of a heterologous protein were controlled for by recording currents from cells that had simply been transfected with the GFP-expressing plasmid. The identity of all cDNA

constructs was independently confirmed by sequencing at the DNA Analysis Facility, Ninewells Hospital and Medical School, University of Dundee.

Western blotting

Protein was extracted from cells on Petri dishes by scraping/sonication in a lysis buffer containing phosphatase and protease inhibitors (1% Triton; 50 mM Tris–HCl, pH 7.5; 1 mM ethylene glycol tetraacetic acid; 1 mM EDTA; 1 mM Na orthovanadate; 10 mM glycerol phosphate; 50 mM NaF; 5 mM Na pyrophosphate; 270 mM sucrose; 0.1% β-mercaptoethanol; 1 Roche Mini Protease Inhibitor tablet per 10 mL). Extracted protein was then diluted (3:1) with 4x NuPage LDS sample buffer (Invitrogen), denatured at 70°C (10 min), and aliquots of denatured protein (normally 12 μg) fractionated on sodium dodecyl sulfate polyacrylamide gels (NuPage Novex Bis-Tris Mini gels, Invitrogen) and blotted onto Hybond-P membranes (Amersham, Buckinghamshire, UK). Total NDRG-1 abundance was then determined by Western analysis using an antibody [31] against the full-length protein, while expression of Thr$^{346/356/366}$-phosphorylated NDRG1 was quantified using a phosphospecific antibody against the SGK1-phosphorylated form of the C-terminal nonapeptide [31]. Preliminary experiments verified that these antibodies stained only a single substantive band in protein extracted from H441 cells. The electrophoretic mobility of this protein indicated a molecular weight of 45 kDa, which accords well with the previously reported value of 43–45 kDa [31]. The phosphorylation of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) at Ser^{133} was similarly quantified using antibodies against total and Ser^{133}-phosphorylated CREB (Upstate, Dundee, UK). Results of such experiments were quantified by densitometry using the Syngene Genegenius (Synoptics) image-capturing system, GeneSnap image-capturing program, and GeneTools densitometry analysis program.

Single-cell electrophysiology

Membrane currents (I_m) were recorded from single cells held under voltage clamp in the perforated patch recording configuration using bath and pipette solutions designed to maintain quasiphysiological ionic gradients [11, 13]. In each experiment, the mean current evoked by driving the holding potential (V_{Hold}) through a series of four ramps (−113 to 87 mV in 2 s) was recorded both under standard conditions and after [Na$^+$]$_o$ had been lowered to 10 mM (N-methyl-D-glucammonium substitution). The current that persisted at low [Na$^+$]$_o$ was then subtracted from the corresponding control current in order to isolate the

[Na$^+$]$_o$-dependent component of I_m (I_{Na}). All reported potentials are corrected for the liquid junction potential between the pipette and bath solutions and membrane potentials (V_m) were inferred from the values of V_{Hold} at which I_m was 0. Values of membrane conductance (G_{Na}) were estimated by regression analysis (i.e., $\Delta I / \Delta V_{Hold}$). These methods are described in detail elsewhere [11, 13].

Electrometric studies

H441 cells grown to confluence on Snapwell membranes in dexamethasone-containing (0.2 μM) medium were mounted in Ussing chambers and bathed with bicarbonate-buffered physiological salt solution so that short circuit current (I_{SC}) could be recorded. To assess the Na$^+$ conductance of the apical membrane $G_{Na(Ap)}$, the salt solution bathing both sides of the cell layer was replaced with a "cytoplasm-like" solution containing gluconate as the principal anion [16]. The basolateral membrane was then permeabilized (100 μM nystatin) and the increase in apical membrane current (ΔI_{Ap}) evoked by imposing an inwardly directed [Na$^+$] gradient upon the basolaterally permeabilized cells was then measured. Since the electrochemical driving force for Na$^+$ entry (V_{Na}) is known (Nernst equation), $G_{Na(Ap)}$ can be determined using the expression $G_{Na} = \Delta I_{Ap} / V_{Na}$. A detailed account of this method is published elsewhere [16, 37].

Results

NDRG1-Thr$^{346/356/366}$ phosphorylation induced by SGK1-S422D

Figure 1 shows data from strictly paired experiments that explored the effects of expressing SGK1-K127A and SGK1-S422D upon the levels of total and Thr$^{346/456/366}$-phosphorylated NDRG1 in hormone-deprived H441 cells. These studies show clearly that the NDRG1 protein is expressed by these cells and it is clear that both groups of cells express very similar levels of the total NDRG1. However, the Thr$^{346/356/366}$-phosphorylated form of NDRG1 was approximately twofold more abundant in the SGK1-S422D-expressing cells (Fig. 1b) and, since this SGK1 mutant displays a constitutively active phenotype [25], this result confirms [31] that increases in cellular SGK1 activity cause increased phosphorylation of NDRG1-Thr$^{346/356/366}$. Moreover, expression of SGK1-S422D caused the Thr$^{346/356/366}$-phosphorylated NDRG1 to migrate as a doublet band and it is interesting in this context that the phosphorylation of NDRG1-Thr$^{346/356/366}$ by SGK1 permits further phosphorylation of this protein by glycogen synthase kinase 3 (GSK3). This GSK3-mediated phosphorylation has been shown to affect the protein's
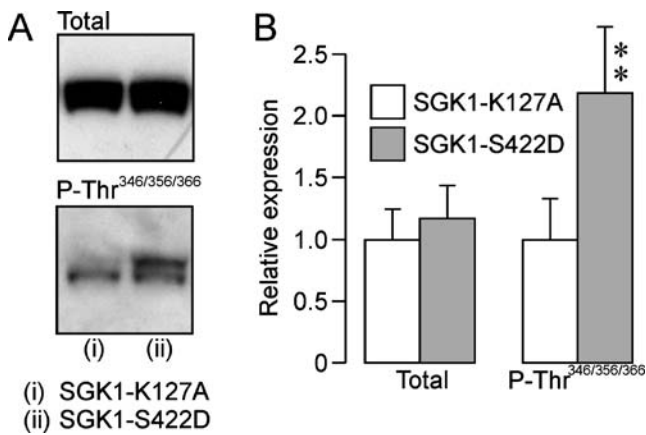
Fig. 1 NDRG1 phosphorylation in SGK1-expressing cells. **a** Western blots showing the levels of total and $Thr^{346/356/366}$-phosphorylated NDRG1 detected in hormone-deprived cells that had been transfected with *p*GL3 plasmids encoding SGK1-K127A or SGK1-S422D. **b** Densitometric analysis of the pooled data (mean±SEM) from five such experiments. All data have been normalized to the mean abundance of total and SGK1-phosphorylated NDRG1 measured in cells expressing the catalytically inactive SGK1-K127A mutant. **$P<0.02$, statistically significant differences in the expression levels measured in cells expressing the two forms of SGK1 (Student's paired *t* test)

electrophoretic mobility and induce the formation of a doublet band [31].
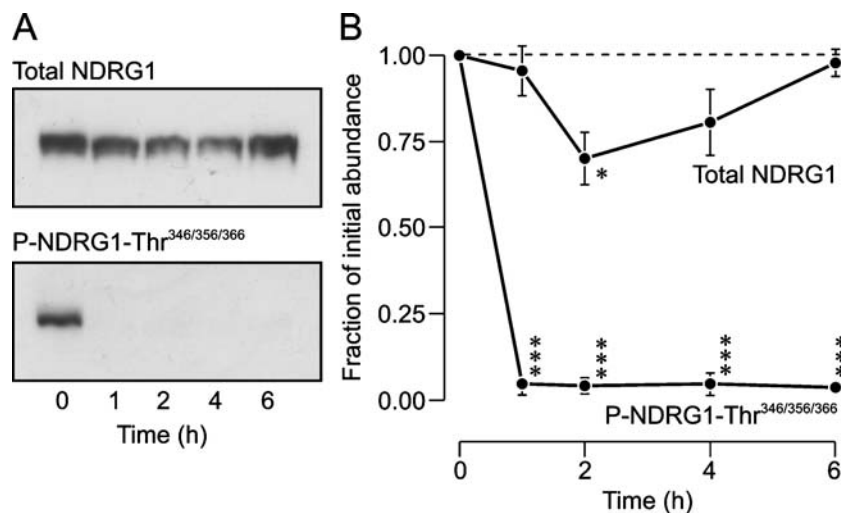
NDRG1-$Thr^{346/356/366}$ phosphorylation in unstimulated cells

Figure 2a (upper panel) shows that the NDRG1 protein is expressed by untransfected H441 cells maintained in hormone-deprived medium, and assays using the phospho-

specific antibody [31] revealed phosphorylation of NDRG1-$Thr^{346/356/366}$ under these conditions (Fig. 2a). LY294002 (50 μM), a PI3K inhibitor, essentially abolished the expression of this phosphoprotein (Fig. 2a) within 1 h and there was no recovery within 6 h (Fig. 2b). Although LY294002 also reduced the total abundance of NDRG1, this effect was relatively small (approximately 25% reduction), developed slowly (approximately 2 h), and was not sustained (Fig. 2a,b). It is, therefore, clear that the LY294002-induced loss of $Thr^{346/356/366}$-phosphorylated NDRG1 cannot be attributed to an effect upon overall expression, and these data, therefore, show that LY294002 causes essentially complete dephosphorylation of NDRG1-$Thr^{346/356/366}$ in hormone-deprived cells.
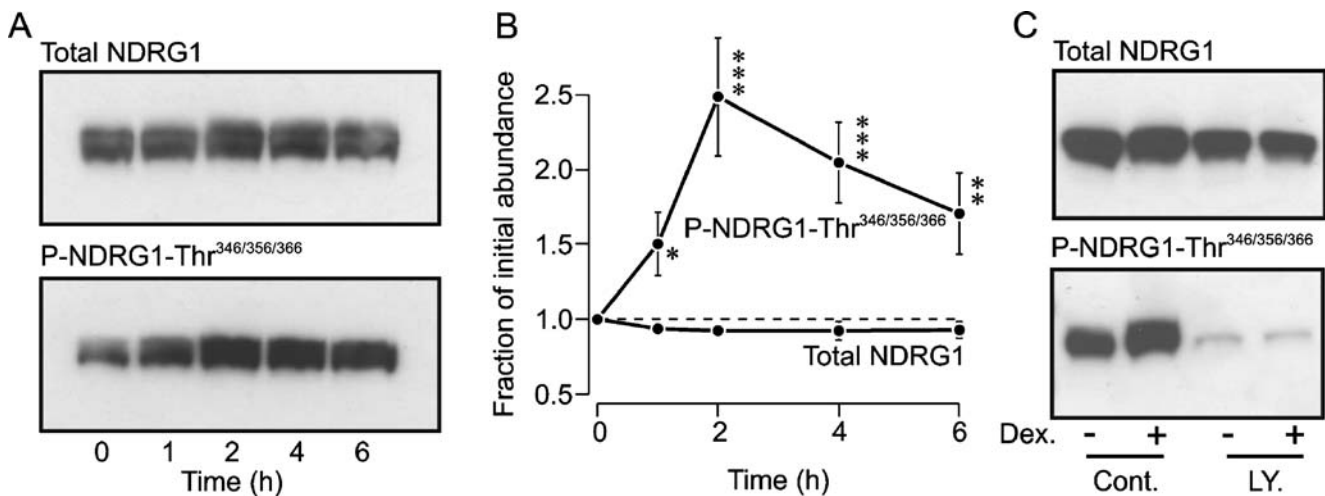
Dexamethasone-induced phosphorylation of NDRG1-$Thr^{346/356/366}$

Acute application of dexamethasone (0.2 μM) increased the abundance of $Thr^{346/356/366}$-phosphorylated NDRG1 with no effect upon total expression (Fig. 3a,b), demonstrating that this synthetic glucocorticoid evokes increased phosphorylation of NDRG1-$Thr^{346/356/366}$. Moreover, in some experiments, dexamethasone caused the $Thr^{346/356/366}$-phosphorylated protein to migrate as a doublet band (Fig. 3a) although this was not a consistent finding. Dexamethasone-induced phosphorylation of NDRG1 was evident after 1 h, peaked after 2–3 h (Fig. 3b), and although there was some decline from this peak, the abundance of the $Thr^{346/356/366}$-phosphorylated protein remained elevated for 6 h (Fig. 3b). Figure 3c shows the results of experiments

Fig. 2 Phosphorylation of NDRG1-$Thr^{346/356/366}$ in unstimulated cells. **a** Typical Western blots showing the effects of LY294002 (50 μM, 0–6 h) upon the overall expression of NDRG1 protein (*upper panel*) and upon the cellular abundance of the $Thr^{346/356/366}$-phosphorylated form of this protein (*lower panel*). **b** Densitometric analysis showing the LY294002-induced changes in the cellular

abundance of total and $Thr^{346/356/366}$-phosphorylated NDRG1. Data have been normalized to the expression level measured at the onset of the experiment and are presented as the mean±SEM ($n=6$). *$P<0.05$; ***$P<0.001$; data points that differed significantly from this initial value

Fig. 3 Acute effects of dexamethasone upon NDRG1-Thr$^{346/356/366}$ phosphorylation. **a** Typical Western blots showing the effects of dexamethasone (0.2 μM) upon the overall abundance of NDRG1 and the abundance of the Thr$^{346/356/366}$-phosphorylated protein in cells that had been maintained in hormone-free medium for 4 days. **b** Densitometric analysis showing the effects of dexamethasone upon the cellular abundance of total and Thr$^{346/356/366}$-phosphorylated NDRG1; data are normalized to the level measured at the onset of each experiment and are presented as the mean±SEM ($n=17$). *$P<$0.05, **$P<$0.02, ***$P<$0.01; data points that differed from the initial values. **c** Western blot showing the effects of LY294002 (50 μM, 2 h) upon the cellular abundance of total and Thr$^{346/356/366}$-phosphorylated NDRG1 in unstimulated and dexamethasone-stimulated (0.2 μM, 2 h) cells. Essentially identical data were obtained in four independent experiments

($n=4$) that explored the effects of LY294002 (50 μM, 2 h). As anticipated (see above), this substance essentially abolished the phosphorylation of NDRG1-Thr$^{346/356/366}$ in unstimulated cells (see Fig. 2) and also blocked the dexamethasone-induced phosphorylation of these residues.

Figure 4 shows the changes in abundance of total and Thr$^{346/356/266}$-phosphorylated NDRG1 induced by long-term (0–7 days) exposure to dexamethasone. The culture medium bathing all cells used in these experiments was changed daily, and control cells were exposed to nominally hormone-free medium throughout the entire experimental period, while the experimental cells were exposed to dexamethasone-supplemented (0.2 μM) medium for the final 1–7 days. All cells were then lysed and protein was extracted for Western analysis. This experimental design ensured that changes in NDRG1 expression cannot be attributed to variations in the time spent in culture. Visual examination of these data suggested that prolonged exposure to dexamethasone reduced the overall abundance of NDRG1 and analysis of variance indicated that the duration of exposure to this hormone did reduce total NDRG1 levels ($P<$0.05). However, analysis of the individual time points did not confirm this effect, although analysis of the pooled data derived from cells that had been exposed to dexamethasone for 4–7 days did reveal significant inhibition (26.7±4.4%, $P<$0.001, Student's t test). Prolonged exposure to dexamethasone, therefore, causes a slight reduction in the overall abundance of the NDRG1 protein. However, prolonged exposure to dexamethasone caused an unambiguous fall in the expression of Thr$^{346/356/366}$-phosphorylated

NDRG1 that developed over 1–3 days (Fig. 4a,b). To define this effect more fully, the abundance of Thr$^{346/356/266}$-phosphorylated NDRG1 was normalized to the total expression at each time point, and this analysis showed that NDRG1-Thr$^{346/356/366}$ phosphorylation fell to approximately 35% of the control level after 3 days exposure to dexamethasone. There was no evidence of recovery (Fig. 4c).

To explore the possibility that the reduced phosphorylation of NDRG1-Thr$^{346/356/366}$ seen during prolonged exposure to dexamethasone may reflect increased activity of phosphoprotein phosphatases, we explored the effects of okadaic acid, a well-documented inhibitor of such enzymes [14, 48], upon this component of the response. These experiments were undertaken using a strictly paired protocol in which cells were maintained in either hormone-free or dexamethasone-containing medium (0.2 μM) for 24 h. The experimental cells were exposed to okadaic acid for the final 4 h of this incubation period while control cells were exposed to solvent vehicle. Parallel studies of age-matched cells at identical passage confirmed (Fig. 3) that brief (2 h) exposure with dexamethasone (0.2 μM) increased NDRG1-Thr$^{346/356/366}$ phosphorylation while data derived from control cells exposed to 0.2 μM dexamethasone for 24 h verified (Fig. 4) that prolonged stimulation led to a suppression of NDRG1-Thr$^{346/356/366}$ phosphorylation. When used at 1 μM, okadaic acid rapidly and consistently induced cell swelling and the experimental cells detached from the culture substrate within 20–30 min. A similar response was usually seen when okadaic acid was
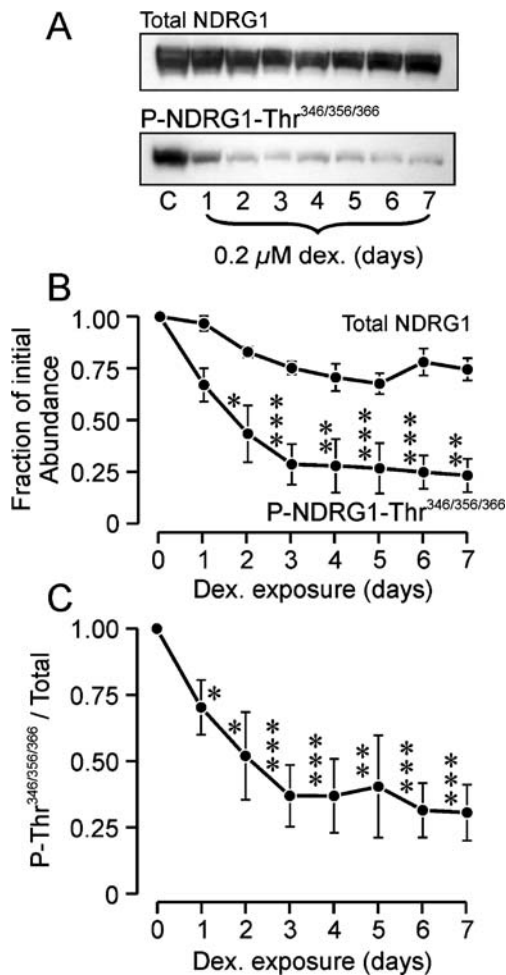
Fig. 4 Changes in NDRG1 phosphorylation induced by long-term exposure to dexamethasone. **a** Typical blots showing the effects of prolonged exposure to dexamethasone upon the abundance of Thr[346/356/366]-phosphorylated NDRG1 and total NDRG1. **b** Densitometric analysis showing the effects of dexamethasone upon the abundance of Thr[346/356/366]-NDRG1 phosphorylated and total NDRG1. **c** Effects of dexamethasone upon the relative phosphorylation of NDRG1 which was determined by normalizing the abundance of the Thr[346/356/366]-phosphorylated protein to the overall abundance measured at each time point. All data are presented as the mean±SEM. *$P<0.05$, **$P<0.02$, ***$P<0.01$; statistically significant variations from the data derived from the cells that were maintained in hormone-free medium for the entire experimental period (analysis of variance/Bonferroni post hoc test)

used at 0.1 μM but this developed more slowly and, in one instance, we did isolate protein from cells that had remained attached to the culture substrate. Analysis of this sample revealed a striking fall in the overall abundance of NDRG1, suggesting that 0.1 μM okadaic acid might stimulate NDRG1 degradation. However, when used at 0.01 μM, okadaic acid had no overt effect upon cell morphology/attachment and analysis of extracted protein ($n=4$) showed that this concentration of okadaic acid also had no effect upon the abundance of either total or Thr[346/356/366]-phosphorylated NDRG in control or dexamethasone-treated cells.

Dexamethasone-induced Na^{+} current

Analysis of the I_m–V_{Hold} relationship for hormone-deprived cells (Fig. 5a) showed that V_m was normally -60.4 ± 3.7 mV and lowering $[Na^+]_o$ had no effect upon V_m (Fig. 5a; $V_m=-61.0\pm4.1$ mV), indicating that G_{Na} is negligible under these conditions [see also 11, 13]. Further analysis confirmed this conclusion by showing that I_{Na} was negligible across the entire range of potentials tested (Fig. 5b). Parallel studies of age-matched cells that had been exposed to 0.2 μM dexamethasone for 2–3 h (Fig. 5c) showed that this hormone depolarized V_m by approximately 35 mV ($V_m=-25.4\pm2.9$ mV; $P<0.01$) and lowering $[Na^+]_o$ consistently hyperpolarized these cells (-52.3 ± 5.3 mV; $P<0.01$). Analysis of these data revealed a dexamethasone-induced, depolarizing Na^{+} current (Fig. 5d) and regression analysis showed that G_{Na} (601 ± 220 pS cell^{-1}) accounted for $33.8\pm7.0\%$ of the total membrane conductance at physiologically relevant potentials. The magnitude of this glucocorticoid-induced conductance is, therefore, essentially identical to that documented in previous studies in which H441 cells were exposed to dexamethasone for 24–48 h [11, 13] and is, therefore, clear that the dexamethasone-induced increase in G_{Na} is fully developed after only 2–3 h. Figure 5e shows currents recorded from cells that had been stimulated with dexamethasone (0.2 μM, 2–3 h) in the presence of LY294002 (50 μM). Since the effects of LY294002 upon PI3K are reversible, this compound was present in all bath solutions used in these experiments. The currents recorded under these conditions were essentially identical to those recorded from hormone-deprived cells (Fig. 5e). Lowering $[Na^+]_o$, therefore, had no effect upon V_m (control: $V_m=-60.7\pm5.2$ mV; low Na^{+}: $V_m=-62.3\pm5.0$ mV) and further analysis showed that I_{Na} was negligible (Fig. 5f). LY294002, therefore, abolishes the dexamethasone-induced increase in G_{Na}.

Figure 6a shows the results of experiments that explored the effects of heterologously expressing SGK1-K127A upon the induction of G_{Na} by relatively brief exposure to dexamethasone (0.2 μM, 2–3 h). Initial studies of cells that had simply been transfected with GFP showed that the expression of this fluorescent protein, which served as a marker of transfection in all such experiments (see the "Materials and methods" section), had no discernible effect upon the electrical properties of cells maintained in nominally hormone-free medium ($n=6$). Lowering $[Na^+]_o$, therefore, had no effect upon V_m (control=-56.4 ± 3.0 mV; low Na[+]=-59.0 ± 3.1 mV) and I_{Na} was negligible (Fig. 6a). Moreover, stimulating such GFP-expressing cells with dexamethasone (0.2 μM, 2–3 h) caused $[Na^+]_o$-dependent depolarization (control=-35.3 ± 4.6 mV; low Na[+]=-53.6 ± 5.6 mV; $P<0.05$) by inducing a current essentially identical
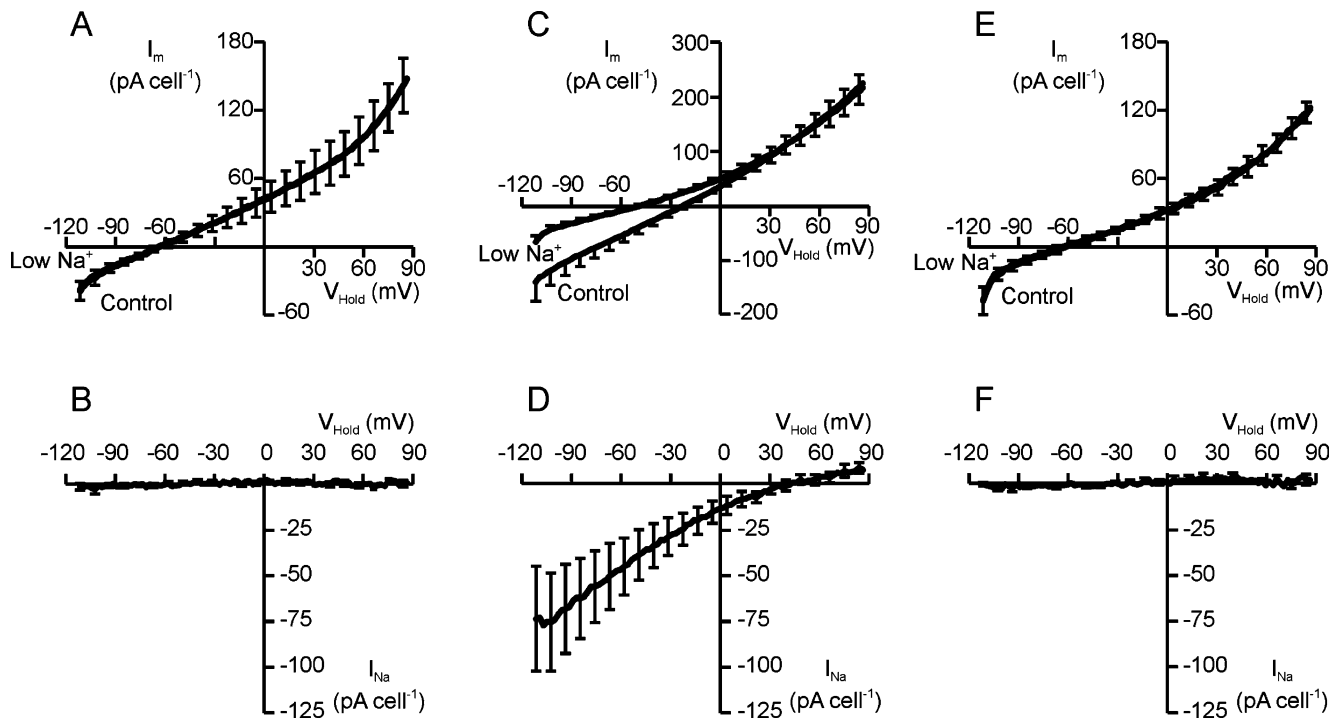
Fig. 5 Dexamethasone-induced Na currents. **a**, **c**, and **e** each show the relationships between membrane current (I_m, mean±SEM) and holding potential (V_{Hold}) and each figure includes data recorded during exposure to the standard bath solution (*Control*) and 20–30 s after $[Na^+]_o$ had been lowered to 10 mM (*Low Na$^+$*). The results of each such experiment were further analyzed by digitally subtracting the current that persisted at low Na$^+$ from the corresponding record of control current in order to isolate the $[Na^+]_o$-dependent component of the total membrane current (I_N), and the results of this analysis are presented (mean±SEM) in **b**, **d**, and **f**. Data were derived from hormone-deprived cells (**a** and **b**, $n=7$); cells stimulated with 0.2 μM dexamethasone for 2–3 h (**c** and **d**, $n=10$), and from cells stimulated with 0.2 μM dexamethasone (2–3 h) in the presence of 50 μM LY294002 (**e** and **f**, $n=6$)

to that seen in untransfected cells (Fig. 6a). It is, therefore, clear that exposure to transfection reagents/expression of heterologous protein does not affect the induction of this current. Analysis of currents recorded from SGK1-K127A-expressing cells that had been stimulated with dexamethasone (0.2 μM, 2–3 h) showed that expression of this catalytically inactive SGK1 mutant had no effect upon V_m (−39.6±4.3 mV) and did not affect the hyperpolarization induced by lowering $[Na^+]_o$ (SGK1-K127A: $\Delta V_m=-21.3\pm2.9$ mV; GFP alone $\Delta V_m=-18.3\pm5.3$ mV) and further analysis showed that this mutant form of SGK1 had no effect upon the dexamethasone-induced I_{Na} (Fig. 6a). It is, therefore, clear that heterologous expression of SGK1-K127A does not disrupt the dexamethasone-mediated control of G_{Na}.

Figure 6b shows the results of experiments that explored the effects of this mutant protein upon the ability of dexamethasone (0.2 μM, 2 h) to induce the phosphorylation of NDRG1-Thr[346/356/366]. Studies of cells that had simply been transfected with the empty *p*GL3 plasmid confirmed that this hormone normally stimulates the phosphorylation of these residues (Fig. 6b). Although SGK1-K127A had no statistically significant effect upon the phosphorylation of NDRG1-Thr[346/356/366] in unstimulated cells, this mutant protein did appear to cause a slight increase the phosphorylation of these residues in some experiments (see Fig. 6b).

However, the most important result to emerge from these studies was that dexamethasone-consistently failed to evoke any further phosphorylation of NDRG1-Thr[346/356/366] in the SGK1-K127A-expressing cells (Fig. 6b). This catalytically inactive form of SGK1, therefore, suppresses this component of the response to dexamethasone without affecting the induction of G_{Na} (Fig. 6a) and this discrepancy suggests that SGK1 may not be important to the dexamethasone-mediated control of G_{Na}. This series of experiments also confirmed (see Fig. 1) that SGK1-S422D consistently increased the phosphorylation of NDRG1-Thr[346/356/366] in hormone-deprived cells and established that dexamethasone has no additional effect these conditions (Fig. 6b).

Forskolin-evoked Na$^+$ transport in confluent cells

As anticipated by earlier work [27, 37, 49], H441 cells cultured on permeable supports and maintained (7 days) in dexamethasone-containing medium became incorporated into electrically resistive epithelial sheets and generated amiloride-sensitive I_{SC} due to electrogenic Na$^+$ absorption. Figure 7a shows that LY294002 (50 μM) caused substantial (approximately 60%) inhibition of this dexamethasone-induced [see, e.g., 37] current [see also 49]. This effect became apparent as soon as the cells were exposed to the
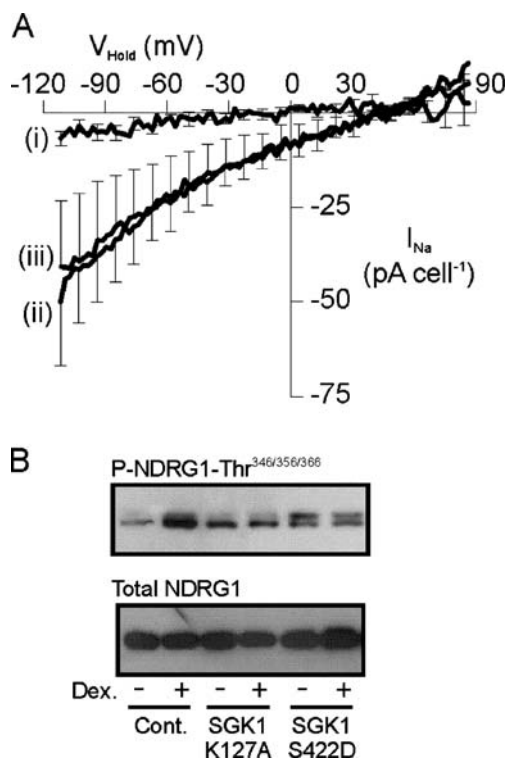
Fig. 6 Effects of SGK1-K127A upon the dexamethasone-induced I_{Na} and phosphorylation of NDRG1-Thr[346/356/366]. **a** In each experiment, I_m was recorded both under standard conditions and after $[Na^+]_o$ had been lowered to 10 mM and these data were then analyzed in order to isolate I_{Na} which has been plotted (mean±SEM) against V_{Hold}. *i* Data derived from GFP-expressing cells maintained in nominally hormone-free medium ($n=6$). *ii* Data from GFP-expressing cells exposed to 0.2 μM dexamethasone for 2–3 h ($n=6$). *iii* Data from cells expressing GFP/SGK1-K127A that had been exposed to 0.2 μM dexamethasone for 2–3 h ($n=6$). **b** Typical blots showing the effects of dexamethasone (0.2 μM, 2 h) upon the abundance of Thr[346/356/366]-phosphorylated and total NDRG1 in cells that had simply been transfected with the empty *p*GL3 plasmid (*Cont.*) and in cells expressing SGK1-K127A or SGK1-S422D. Essentially identical; results were obtained in five independent experiments

drug but developed relatively slowly (20–30 min; Fig. 7a). Once this inhibitory action was fully developed, the cells were basolaterally permeabilized so that $G_{Na(Ap)}$ could be measured (see the "Materials and methods" section) and the results of this analysis are shown in Fig. 7b together with data from age-matched cells that had not been exposed to LY294002 but which had otherwise been treated identically. These data show that the LY294002-induced fall in I_{SC} was associated with a clear reduction in $G_{Na(Ap)}$ (Fig. 7b). Figure 7a also shows that forskolin, a diterpene that evokes cAMP formation [42], increased the I_{SC} recorded from dexamethasone-stimulated cells and studies of basolaterally permeabilized cells confirmed [37] that this response is associated with a rise in G_{Na} (Fig. 7b). Moreover, forskolin consistently increased I_{SC} (Fig. 7a) and $G_{Na(Ap)}$ (Fig. 7b) in LY294002-treated cells and so, although this inhibitor of

PI3K inhibits the dexamethasone-induced I_{SC}, it does not block the electrometric response to forskolin [see also 49].

Forskolin-induced phosphorylation of CREB-Ser[133] and NDRG1-Thr[346/356/366]

Analyses of protein extracted from cells grown to confluence in dexamethasone-containing medium (8 days) showed that forskolin (10 μM, 20 min) evoked phosphorylation of CREB-Ser[133], and this response, which is consistent with increased activity of protein kinase A (PKA) [42], persisted in LY294002-treated cells (Fig. 7c,d). Visual examination of these data suggested that LY294002 might slightly increase the phosphorylation of CREB-Ser[133] (Fig. 7c,d) but this effect was not statistical significant in this series of experiments (Fig. 7d). However, analysis of the pooled data from all experiments in which we monitored the phosphorylation of CREB-Ser[133] in the presence of LY294002 (i.e., including the data shown in Fig. 8) showed that this PI3K inhibitor did evoke a small increase (21.7± 6.4% stimulation, $P<0.05$) in CREB-Ser[133] phosphorylation. This suggest that PI3K-dependent and cAMP-dependent signaling pathways may interact, but the physiological basis of this effect was not investigated further in the present study. Although prolonged stimulation with dexamethasone reduced the phosphorylation of NDRG1-Thr[346/356/366], analysis of protein extracted from cells that had been chronically exposed to this hormone could still provide a clear signal on a Western blot if the amount of protein loaded onto the gels was increased (25 μg) and if the exposure time was extended. Such analyses showed that LY294002 essentially abolished the residual phosphorylation of NDRG1-Thr[346/356/366] that persisted under these conditions, and established forskolin had no discernible effect upon the phosphorylation of NDRG1-Thr[346/356/366] in either control or LY294002-treated cells (Fig. 7e,f). The electrometric response to forskolin, therefore, occurs with no change in SGK1 activity.

Insulin-induced Na^{+} transport in confluent cells

It is well-established that insulin stimulates Na^{+} transport in the epithelia of the distal nephron [see, e.g., 5, 7] and this hormone has recently been shown to augment the dexamethasone-induced G_{Na} in H441 cells [11]. The present study, therefore, explored the effects of insulin upon the I_{SC} generated by H441 cells grown to confluence in dexamethasone-containing medium. Figure 8a shows that this hormone (20 nM) acutely increased the I_{SC} recorded under these conditions and, although this response was preceded by a transient fall in I_{SC}, it was generally similar to the response to forskolin (see Fig. 7a). Studies of basolaterally permeabilized cells showed that this response to insulin was associated with a rise in $G_{Na(Ap)}$ (Fig. 8b), and it is,

Fig. 7 The response to forskolin in confluent cells. **a** Results of electrometric experiments ($n=6$) showing the effects of forskolin (10 μM, added as indicated by the *shaded box*) upon the I_{SC} generated and LY294002-treated cells (50 μM, added as indicated by the *arrow*). Control cells were exposed to solvent vehicle. **b** Effects of forskolin upon G_{Na} in control and LY294002-treated cells. *Shaded bars* show data from forskolin-stimulated cells while *open bars* show data from unstimulated, age-matched cells at identical passage. **c** Typical Western blots showing the effects of forskolin and LY294002 upon the total abundance of CREB (*upper panel*) and the abundance of phosphorylated CREB (*lower panel*). **d** Densitometric analysis showing the pooled results of five independent experiments. **e** Typical Western blots showing the effects of forskolin and/or LY294002 upon the overall abundance of NDRG-1 (*upper panel*) and the abundance of phosphorylated NDRG-1 (*lower panel*). **f** Densitometric analysis showing the pooled data from five independent experiments. All values are presented as the mean±SEM. **$P<0.02$, ***$P<0.01$, statistically significant effects of forskolin; †$P<0.05$, statistically significant effects of LY294002 (Student's paired t test)
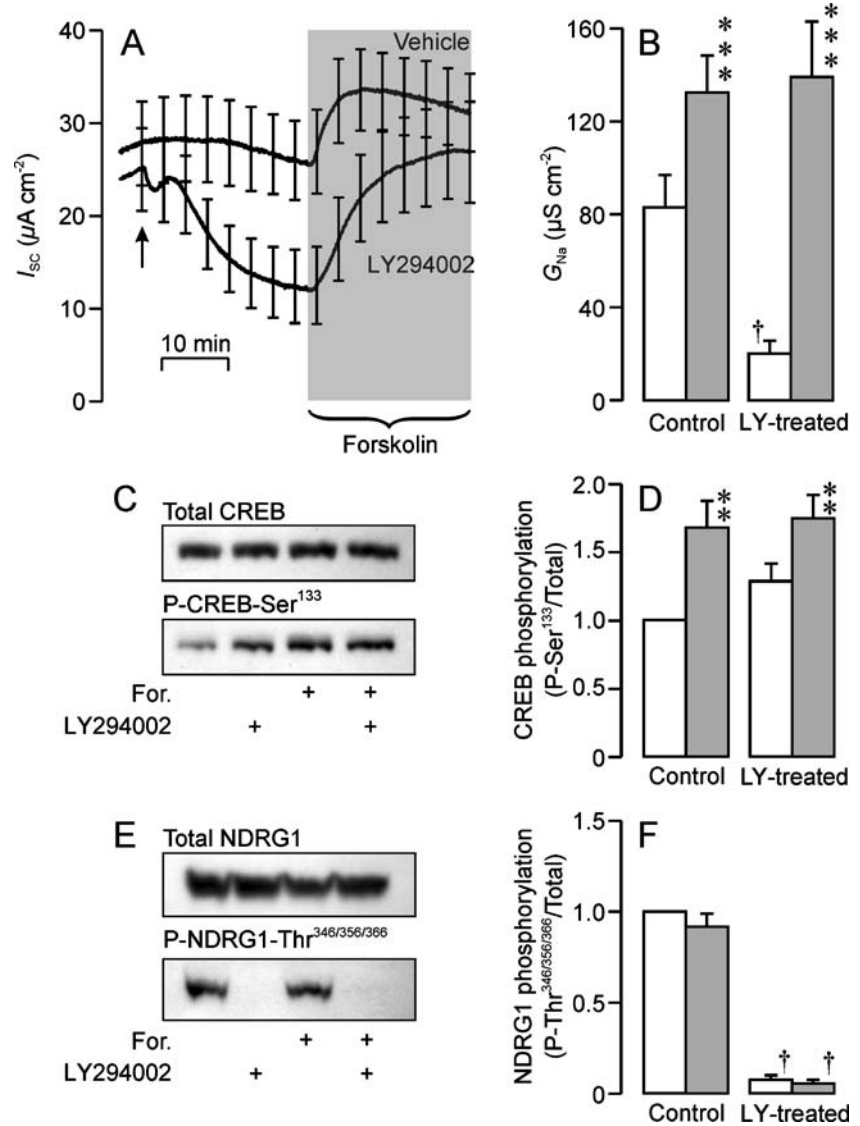
therefore, clear that insulin and forskolin can both acutely regulate the dexamethasone-induced apical Na$^+$ conductance in H441 cells. Figure 8a includes data from age-matched cells at identical passage that had been exposed to LY294002. These data confirm that this PI3K inhibitor reduces the basal (i.e., dexamethasone-induced) I_{SC} but also show that insulin cannot increase I_{SC} (Fig. 8a) or $G_{Na(Ap)}$ (Fig. 8b) in LY294002-treated cells. The electrometric response to insulin, in contrast to the response to forskolin, is, therefore, abolished by LY294002.

Insulin-induced phosphorylation of CREB-Ser[133] and NDRG1-Thr[346/356/366]

Analyses of protein extracted from dexamethasone-treated cells (0.2 μM, 7 days) showed that insulin (20 nM, 30 min)

had no effect upon the phosphorylation of CREB-Ser[133] in either control or LY294002-treated cells (Fig. 8c,d), and this finding is, therefore, consistent with a body of data which show that this hormone does not signal via cAMP/PKA [15]. However, insulin (20 nM, 30 min) normally increased the phosphorylation of NDRG1-Thr[346/356/366] and this response, in common with the stimulation of Na$^+$ transport, was abolished by LY294002 (Fig. 8e,f).

Discussion

NDRG1-Thr[346/356/366] phosphorylation as an SGK1 assay

While the hormonally induced SGK1 expression can be quantified by Western analysis of extracted protein, this

Fig. 8 The response to insulin in confluent cells. **a** Effects of insulin (20 nM, $n=7$, added as indicated by the *shaded box*) upon the I_{SC} generated by control (i.e. solvent vehicle) and LY294002-treated (50 μM, added as indicated by the *arrow*) cells. **b** Effects of insulin upon G_{Na} in control and LY294002-treated cells. *Shaded bars* show data from insulin-stimulated cells while *open bars* show data from unstimulated, age-matched cells at identical passage. **c** Typical Western blots showing the effects of insulin and/or LY294002 upon the overall abundance of CREB (*upper panel*) and the abundance of phosphorylated CREB (*lower panel*). **d** Densitometric analysis of the pooled data from six independent experiments. **e** Typical Western blots showing the effects of insulin and/or LY294002 upon the overall abundance of NDRG-1 (*upper panel*) and the abundance of Thr[346/356/366]-phosphorylated NDRG-1 (*lower panel*). **f** Densitometric analysis showing the pooled data from five independent experiments. All data are presented as the mean±SEM. **$P<0.02$, ***$P<0.01$, statistically significant effects of insulin; †$P<0.05$, statistically significant effects of LY294002 (Student's paired t test)
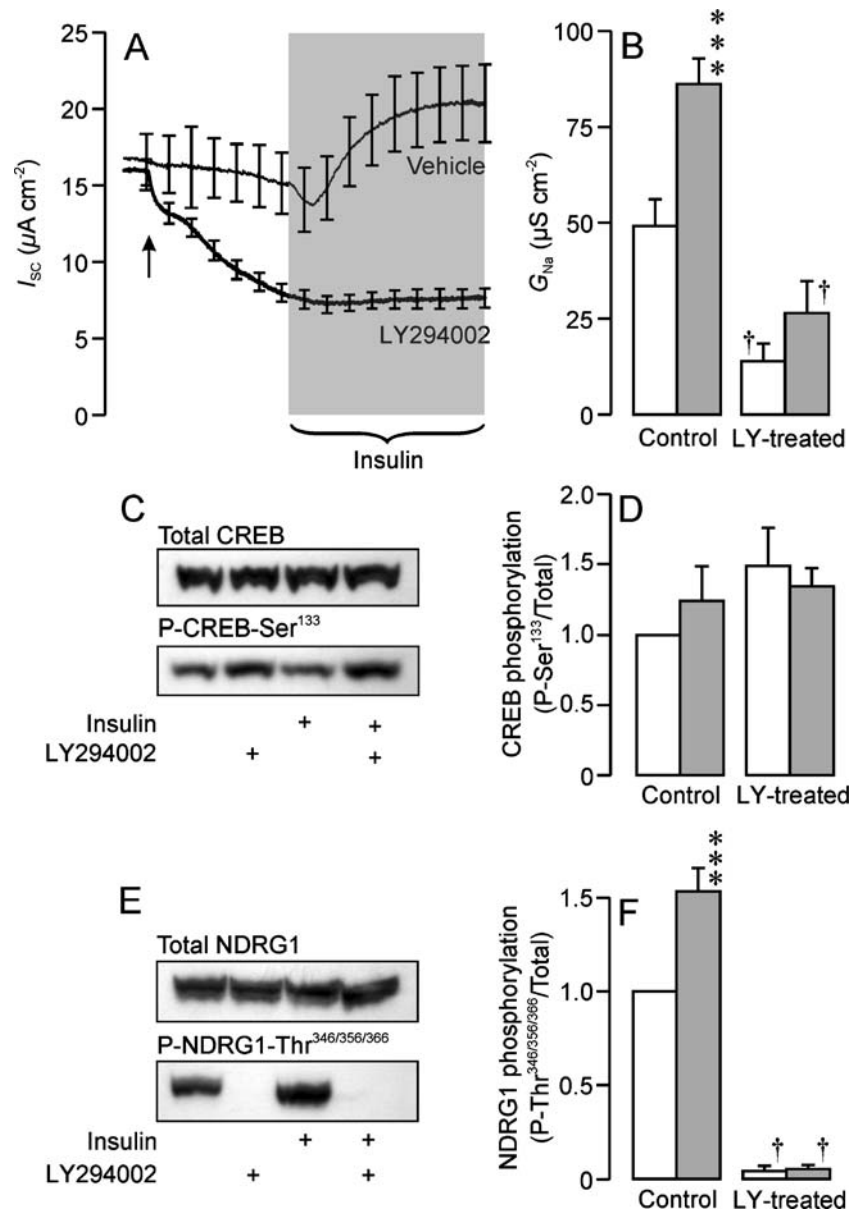
approach is complicated by the fact that SGK1 is expressed at very low levels in many cell types, implying that large amounts of protein are needed to obtain meaningful signals. Demonstration of dexamethasone-induced SGK1 expression in A6 cells [51] thus required the fractionation of 105 μg aliquots of cellular protein while some of the analyses presented in an earlier study of H441 cells [49] involved the fractionation of 400 μg protein aliquots. Moreover, although the activation of SGK1 has been studied using phosphospecific antibodies to monitor the phosphorylation of specific residues within this protein, this approach has usually been used to study the control of heterologously expressed SGK1 [e.g., 21]. When used to assess the phosphorylation of endogenous SGK1, these antibodies almost invariably stain multiple bands which

makes the results of such studies hard to interpret [for example, see Fig. 9 in ref. 31].

There is, therefore, a need for a simple and relatively sensitive method of assaying cellular SGK1 activity and studies of HeLa cells and SGK1 knockout mice showed that the phosphorylation of NDRG1-Thr[346/356/366] can provide a read out of the activity of this kinase [31]. These studies showed clearly that the phosphorylation of NDRG1-Thr[346/356/366] is entirely dependent upon the expression of SGK1 [31] and stimuli that characteristically activate SGK1 (exposure to serum, stimulation with insulin-like growth factor 1) thus have no effect upon the phosphorylation of these residues if cellular SGK1 expression is abolished using short interfering RNAs. However, such knockdown of SGK1 has no effect upon the activation of PKB, a closely related kinase that is

often activated in parallel with SGK1 [15]. While these data show that NDRG1-Thr$^{346/356/366}$ is phosphorylated by SGK1 but not by other related kinases [31, 32], the functional significance of this phosphorylation is unknown. As far as we are aware, there is no evidence that NDRG1 mediates any SGK1-dependent response, although a recent study has provided very strong evidence to suggest that the closely related NDRG2 protein, which is also phosphorylated by SGK1 [31], may be directly involved in the hormonal stimulation of epithelial Na$^+$ channel function [52].

The fact that NDRG1-Thr$^{346/356/366}$ phosphorylation is so strictly dependent upon SGK1 suggested that changes in cellular SGK1 activity might be monitored by assaying the phosphorylation status of these residues. The present data further support this hypothesis by showing that transfection with a plasmid encoding SGK1-S422D, an SGK1 mutant that displays an active phenotype [25], increased the phosphorylation of NDRG1-Thr$^{346/356/366}$ to a level approximately twofold above that measured in cells expressing a catalytically inactive, control construct (SGK1-K127A). Moreover, analysis of protein extracted from hormone-deprived cells showed that the basal phosphorylation of NDRG1-Thr$^{346/356/366}$ was abolished by LY294002, an inhibitor of PI3K [see also 31]. This provides further support for the view that NDRG1-Thr$^{346/356/366}$ phosphorylation can indicate cellular SGK1 activity since the activity of SGK1 is maintained by PI3K, which controls the phosphorylation of SGK1-Ser^{422} and SGK1-Ser^{256} by PDK2 and PDK1, respectively [25, 34]. It thus appears that this new method [31] may provide a relatively simple way of monitoring the activity of SGK1 in Na$^+$-absorbing epithelia, and the present study, therefore, used this new approach to explore the role of SGK1 in the induction/regulation of the glucocorticoid-dependent G_{Na} in H441 human airway epithelial cells [11, 13, 37].

Dexamethasone-induced activation of SGK1

Acute exposure to dexamethasone evoked phosphorylation of NDRG1-Thr$^{346/356/366}$, indicating increased SGK1 activity, and this finding accords with the well-documented properties of this kinase [26] and with data from earlier studies of Na$^+$-absorbing epithelia which showed that dexamethasone increases the expression of SGK1 mRNA and protein [24, 49, 51]. The relative sensitivity of the assay used in this study [31] allowed us to show that the activation of SGK1 is apparent after approximately 1 h and peaks at 2–3 h. Moreover, this response was abolished by LY294002, indicating a requirement for PI3K [see also 25, 34], and it is, therefore, interesting to note that earlier studies of A6 cells showed that LY294002 does not block steroid-induced synthesis of SGK but does abolish the subsequent phosphorylation of this protein [34, 51].

We also explored the changes in SGK1 activity evoked by prolonged exposure to dexamethasone and these data suggested that the increased activity of this enzyme is not sustained since, after 3 days, the phosphorylation of NDRG1-Thr$^{346/356/366}$ had fallen to approximately 30% of the level measured in hormone-deprived cells. Although the earlier assays of SGK1 mRNA and protein levels similarly suggested that dexamethasone-induced SGK1 activation might not be well-maintained [24, 49, 51], the present data were still surprising since we can routinely record dexamethasone-induced Na$^+$ currents from H441 cells that have been exposed to this hormone for 24–48 h [11, 13], and the present data suggest that SGK1 activity may actually be reduced by this time. Moreover, cells grown to confluence in dexamethasone-containing medium for 6–8 days consistently generate I_{SC} due to the expression of a glucocorticoid-induced apical Na$^+$ conductance [27, 37] and the present data suggest that this dexamethasone-induced absorption of Na$^+$ in H441 cells may persist despite a loss of cellular SGK1 activity.

We explored the possibility that this loss of NDRG1 phosphorylation may reflect increased activity of phospho-protein phosphatases (PP1, PP2A, etc.) by studying the effect of okadaic acid, a substance that inhibits such enzymes [14, 48]. However, the interpretation of these experiments was complicated by the fact that the cells did not tolerate okadaic acid at concentrations high enough to achieve unambiguous inhibition of phosphoprotein phosphatase activity. However, the dexamethasone-induced loss of NDRG1-Thr$^{346/356/366}$ phosphorylation was not reversed by 0.01 μM okadaic acid and this concentration is high enough to cause essentially complete inhibition of PP2A and PP4 and to substantially reduce the activity of PP5 [14, 48]. While these enzymes do not appear to mediate the dephosphorylation of NDRG1 during prolonged exposure to dexamethasone, we cannot exclude the possibility that enzymes such as PP1 and PP2B may underlie the dephosphorylation of NDRG1-Thr$^{346/356/366}$ that develops during prolonged exposure to dexamethasone [14, 48].

Dexamethasone-induced I_{Na}

While stimulation with dexamethasone depolarizes hormone-deprived H441 cells by increasing G_{Na} [11, 13], the signaling pathways underlying this response are not well-defined. However, studies of other Na$^+$-absorbing epithelia have shown that G_{Na} is normally restricted by the continual removal of Na$^+$ channels from the plasma membrane and this channel internalization process seems to be mediated by Nedd-4/2, a ubiquitin ligase which binds to Na$^+$ channels in the plasma membrane and targets them for ubiquitination, internalization, and degradation [17, 44, 47]. In this context, it is relevant that SGK1 can block this

interaction between Nedd-4/2 and the epithelial Na^+ channel by phosphorylating Nedd-4/2 at Ser^{221}, Thr^{246}, and Ser^{327}. This allows SGK1 to control G_{Na} by regulating Na^+ channel internalization, and heterologous expression of active SGK1, therefore, mimics the effects of steroid stimulation by activating the endogenous Na^+ conductance in A6 cells and in H441 cells [1, 11, 19] and also enhances I_{Na} in *Xenopus* oocytes and fisher rat thyroid cells expressing the component subunits of the epithelial Na^+ channel [12, 29, 33, 45]. However, it is interesting to note that Nedd-4/2 also mediates the ubiquitination/degradation of SGK1 itself and, although the SGK1-mediated phosphorylation of Nedd-4/2 blocks the interaction with the epithelial Na^+ channel, this phosphorylation does appear to facilitate an interaction between Nedd-4/2 and SGK1 [56]. This mechanism, therefore, allows SGK1 to control its own degradation [56] and this negative feedback phenomenon could contribute to the apparent loss of SGK1 activity seen during prolonged exposure to dexamethasone (Fig. 4).

The fact that SGK1 can influence Na^+ channel internalization/degradation suggested that the activation of SGK1 by steroid hormones can explain the associated rise in G_{Na}, and the present data support this hypothesis by showing that the dexamethasone-induced rise in G_{Na} coincides with the activation of SGK1. Moreover, the rise in G_{Na} and the activation of SGK1 are both abolished by LY294002 and this accords well with earlier data from A6 cells which showed that inhibition of PI3K blocks aldosterone-induced Na^+ transport by reducing the number of active Na^+ channels in the apical membrane [6, 35]. It is interesting to note in this context that, as well as phosphorylating Nedd-4/2, SGK1 has also been shown to evoke increased epithelial Na^+ channel activity by a separate mechanism dependent upon the phosphorylation of a residue (Ser^{621}) within an SGK1 consensus sequence in the channel's α-subunit [18].

However, not all of our data were consistent with the idea that SGK1 played such a central role in the control of G_{Na} since the expression of a catalytically inactive SGK1 mutant (SGK1-K127A) that has been shown to exert a dominant negative effect in *Xenopus* oocyte expression systems [29] did not affect the induction of G_{Na} by dexamethasone and, while this result was initially surprising, it does agree with earlier data from A6 cells. Moreover, the expression of SGK1-K127A expression did suppress the dexamethasone-induced phosphorylation of NDRG1-$Thr^{346/356/366}$, suggesting that this mutant protein does suppress the hormonally induced activation of SGK1. The present data also show hormone-deprived H441 display basal SGK1 activity despite the fact that G_{Na} is negligible [present study, 11, 13] and, taken together, these observations provide evidence against the idea that SGK1 allows dexamethasone to control Na^+ transport. It is important to remember that SGK1 is not the only steroid-inducible gene involved in the control of G_{Na} since glucocorticoids also regulate the transcription of genes encoding the epithelial Na^+ channel subunits [10, 41] and induce expression of a protein known as glucocorticoid-inducible leucine zipper protein that suppresses signaling via the extracellular signal-regulated kinase (ERK)-dependent pathway [4]. This latter action could be highly significant since the activation of ERK is known to inhibit epithelial channel expression and function [8, 28, 43, 54, 55].

Regulation of Na^+ transport in confluent cells

The present data confirm that H441 cells grown to confluence on permeable supports in dexamethasone-containing medium become incorporated into electrically resistive epithelial sheets that absorb Na^+ from the apical bath [see 27, 37, 49]. Although prolonged exposure to dexamethasone caused a fall in NDRG1-$Thr^{346/356/366}$ phosphorylation (see above), LY294002 virtually abolished the residual phosphorylation, indicating almost total inactivation of SGK1. LY294002 also reduced I_{SC} and $G_{Na(Ap)}$, and these findings are consistent with the view that PI3K is important to the maintenance of cellular SGK1 activity [25, 34] and to steroid-induced Na^+ transport [6, 49]. The studies also confirm [27, 37, 49] that forskolin stimulates transepithelial Na^+ transport and extend upon these earlier findings by showing that insulin induces a similar response. Both responses involved increases in $G_{Na(Ap)}$ and, as far as we are aware, these data provide the first demonstration that insulin can control transepithelial Na^+ transport in H441 cells although this is well-documented in renal epithelia [e.g., 1, 38].

Forskolin characteristically evokes signaling via cAMP/PKA [42], and studies of granulosa cells [20] and COS-7 cells [36] suggest that the activation of this signaling pathway may lead to increased activity of SGK1. Moreover, forskolin has been reported to increase the phosphorylation of SGK1 in H441 cells and it was, therefore, suggested that cAMP/PKA-induced Na^+ transport might involve increased activity of SGK1 [36, 49]. However, the apparent effect of forskolin upon SGK1 phosphorylation developed rather slowly, becoming evident after approximately 15 min and increasing progressively over the following 45 min [49] while forskolin-evoked Na^+ transport is a much more rapid response that is evident after only 3–5 min [see, e.g., present study, 49]. Moreover, the present data show that forskolin-evoked Na^+ transport was not associated with any change to the phosphorylation status of NDRG1-$Thr^{346/356/366}$ and also established that this response persists in LY294002-treated cells despite essentially complete loss of SGK1 activity/NDRG1-$Thr^{346/356/366}$ phosphorylation. The response to forskolin was, however,

associated with increased phosphorylation of CREB-Ser^{133} and, since the phosphorylation of this residue indicates PKA activity, our data suggest that the activation of the cAMP/PKA-evoked Na$^+$ transport occurs independently of any change in SGK1 activity. Insulin-evoked Na$^+$ transport, on the other hand, was associated with increased phosphorylation of NDRG1-Thr$^{346/356/366}$ and was very effectively abolished by LY294002. These data accord well with the idea that insulin characteristically activates SGK1 [15], and it is, therefore, interesting to note that studies of renal epithelia have also implicated SGK1 in the control of insulin-evoked Na$^+$ transport [see, e.g., 5, 7, 38]. Indeed, genetic variance in the *sgk1* gene is associated with insulin-dependent hypertension that appears to be secondary to increased Na$^+$ transport in the aldosterone-sensitive epithelia of the distal nephron [22, 23, 50].

Although the electrophysiological responses to forskolin and insulin are superficially similar, it thus appears that the response to insulin is mediated via SGK1 while the response to forskolin is not [see also 5, 7, 38]. It is, therefore, interesting to note that PKA and SGK1 have recently been shown to phosphorylate the same residues on Nedd-4/2, suggesting that this ubiquitin ligase may form an important convergence point between the SGK1-dependent and PKA-dependent signaling pathways [46].

Significance of present findings

The present data, therefore, show that the dexamethasone-induced activation of SGK1 coincides with the increase in G_{Na} and demonstrate that both responses are dependent upon PI3K. While these data suggest that SGK1 might allow steroid hormones to regulate Na$^+$ absorption, we also show (1) that hormone-deprived cells display significant SGK1 activity despite the fact that G_{Na} is negligible, (2) that chronic exposure to dexamethasone causes an apparent loss of SGK1 activity even though Na$^+$ transport is well-maintained [11, 27, 37], and (3) that a catalytically inactive SGK1 mutant does not impair the dexamethasone-induced rise in G_{Na} but does appear to suppress the activation of SGK1. Taken together, these data suggest that the SGK1 may play a minor role in the control of Na$^+$ transport by steroid hormones [see also 1, 2] which may explain why *sgk1* knockout has variable effects in different tissues [39, 53].

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