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RESEARCH PAPER

Epithelial Na^+ channel activity in human airway epithelial cells: the role of serum and glucocorticoid-inducible kinase 1

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BACKGROUND AND PURPOSE

Glucocorticoids appear to control Na^+ absorption in pulmonary epithelial cells via a mechanism dependent upon serum and glucocorticoid-inducible kinase 1 (SGK1), a kinase that allows control over the surface abundance of epithelial Na^+ channel subunits (α-, β- and γ-ENaC). However, not all data support this model and the present study re-evaluates this hypothesis in order to clarify the mechanism that allows glucocorticoids to control ENaC activity.

EXPERIMENTAL APPROACH

Electrophysiological studies explored the effects of agents that suppress SGK1 activity upon glucocorticoid-induced ENaC activity in H441 human airway epithelial cells, whilst analyses of extracted proteins explored the associated changes to the activities of endogenous protein kinase substrates and the overall/surface expression of ENaC subunits.

KEY RESULTS

Although dexamethasone-induced (24 h) ENaC activity was dependent upon SGK1, prolonged exposure to this glucocorticoid did not cause sustained activation of this kinase and neither did it induce a coordinated increase in the surface abundance of α-, β- and γ-ENaC. Brief (3 h) exposure to dexamethasone, on the other hand, did not evoke Na^+ current but did activate SGK1 and cause SGK1-dependent increases in the surface abundance of α-, β- and γ-ENaC.

CONCLUSIONS AND IMPLICATIONS

Although glucocorticoids activated SGK1 and increased the surface abundance of α-, β- and γ-ENaC, these responses were transient and could not account for the sustained activation of ENaC. The maintenance of ENaC activity did, however, depend upon SGK1 and this protein kinase must therefore play an important but permissive role in glucocorticoid-induced ENaC activation.

Abbreviations

ENaC, epithelial sodium channel; I_{Amil}, amiloride-sensitive component of the total membrane current; $I_{Amil\ (-82\ mV)}$, amiloride-sensitive membrane current flowing at –82 mV; I_m, total membrane current; Nedd-4/2, neural precursor cell expressed, developmentally down-regulated protein 4-2; NDRG1, protein encoded by n-myc downstream regulated gene 1; P70-S6K, 70 kDa ribosomal S6 kinase; PDK1, 3-phosphoinositide-dependent protein kinase 1; PIP_2, phosphatidylinostiol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B (also known as Akt); PRAS40, 40 kDa proline-rich substrate of Akt (PKB); SGK1, serum and glucocorticoid-inducible kinase 1; TORC1 and 2, target of rapamycin complex 1 and 2; V_{Hold}, holding potential; V_m, membrane potential

Introduction

The controlled absorption of Na^+ and water from the liquid film that covers the lung and airway epithelia is critical to the integrated functioning of the respiratory tract, and this process depends upon epithelial Na^+ channels (ENaC), transport proteins composed of three subunits (α-, β- and γ-ENaC) that form the selective channels that allow electrogenic Na^+ entry in absorptive epithelia (Canessa *et al.*, 1993; 1994; Knowles and Boucher, 2002; Matthay *et al.*, 2002; Boucher, 2007; channel nomenclature follows Alexander *et al.*, 2011). Glucocorticoids are physiologically important regulators of this pulmonary Na^+ transport process (see Matalon and O'Brodovich, 1999; Matthay *et al.*, 2002; Olver *et al.*, 2004) and such steroid hormones appear to control ENaC via a mechanism dependent upon phosphoinositide-3-kinase (PI3K), a regulatory kinase that catalyses the formation of phospholipid second messengers, primarily phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphatidylinositol-3,5-bisphosphate (PIP_2). Early studies of renal epithelia showed that PI3K inhibitors suppressed Na^+ absorption, apparently by reducing the number of functional Na^+ channels in the apical membrane (Păunescu *et al.*, 2000). This control over the surface abundance of α-, β- and γ-ENaC is now attributed to serum and glucocorticoid-inducible kinase 1 (SGK1), a PI3K-dependent protein kinase that appears able to inhibit the removal of ENaC subunits from the plasma membrane. Activation of SGK1 is therefore thought to allow α-, β- and γ-ENaC to remain in the membrane leading to a rise in its Na^+ conductance and increased Na^+ absorption (Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; De La Rosa *et al.*, 1999; 2003; Păunescu *et al.*, 2000; Debonneville *et al.*, 2001). However, not all data support this hypothesis since *sgk1* gene deletion causes only mild dysfunction of renal Na^+ handling without preventing the hormonal control of colonic Na^+ absorption, and also fails to induce an overt pulmonary phenotype (Wulff *et al.*, 2002; Rexhepaj *et al.*, 2006; Fejes-Toth *et al.*, 2008; Lang *et al.*, 2009). Moreover, pharmacological inhibition of PI3K and/or SGK1 causes only modest (10–20%) inhibition of basal Na^+ transport in mouse cortical collecting duct cells (Mansley and Wilson, 2010a,b).

The role of PI3K/SGK1 in the control of ENaC activity is therefore not fully understood (Lang *et al.*, 2009; Loffing and Korbmacher, 2009) and, to some extent, this reflects the fact that the PI3K inhibitors used in earlier studies (LY294002, wortmannin) are now known to exert many non-specific actions (Bain *et al.*, 2007). Moreover, whilst it was clear that the activation of SGK1 by PI3K is dependent upon phosphoinositide-dependent protein kinase 1 (PDK1), a PI3K-dependent protein kinase that catalyses the phosphorylation of SGK1 at Thr^{256} (Kobayashi and Cohen, 1999; Park *et al.*, 1999; Bayascas and Alessi, 2005), it was also known that this reaction required the prior phosphorylation of SGK1 at Ser^{422} and the kinase responsible for this critical event was unknown (Kobayashi and Cohen, 1999; Park *et al.*, 1999). This situation has now been changed by the development of a new generation of selective inhibitors of PI3K, such as PI103 (see Raynaud *et al.*, 2007) and SGK1 (GSK659034; see Sherk *et al.*, 2008) and by the identification of the target of rapamycin signalling complex 2 (TORC2) as the kinase responsible for the phosphorylation of SGK1 at Ser^{422} (García-Martínez and Alessi, 2008; Lu *et al.*, 2010). Moreover, pharmacological inhibitors of TORC2 are also available (TORIN1, see Thoreen *et al.*, 2009) and these provide an effective means of inactivating SGK1 (García-Martínez and Alessi, 2008; Thoreen *et al.*, 2009; Mansley and Wilson, 2010a).

The present study takes advantage of these developments in order to evaluate the role of PI3K – TORC2 – SGK1 pathway in the glucocorticoid-induced activation of ENaC in an epithelial cell line (H441) derived from the human distal airway (see Lazrak and Matalon, 2003; Clunes *et al.*, 2004; Ramminger *et al.*, 2004; Brown *et al.*, 2008; Lazrak *et al.*, 2009; Althaus *et al.*, 2010).

Methods

Cell culture

H441 cells were routinely maintained in RPMI medium supplemented with 8.5% foetal bovine serum (FBS), 8.5% newborn calf serum, 2 mM glutamine, 5 $\mu g \cdot mL^{-1}$ transferrin, 5 $ng \cdot mL^{-1}$ selenium and an antibiotic/antimycotic mixture (Sigma Chemical Co., Poole, Dorset, UK). For experimentation, cells were removed from culture flasks (trypsin/EDTA) and were plated onto six-well plates (for analyses of extracted protein) or glass cover slips (for electrophysiological studies). Cells on six-well plates were grown to ~80% confluence (5–6 days) before the growth medium was replaced with a fully defined medium identical to that described earlier except that it contained 20 nM insulin and the serum components were replaced with FBS (8.5%) that had been dialysed to remove hormones/growth factors. Glucocorticoid-deprived cells were maintained in this medium for ~24 h before being used in experiments while dexamethasone-treated cells were exposed to medium containing 0.2 μM dexamethasone, as detailed in the text. Cells used in electrophysiological experiments were maintained in standard growth medium until cellular attachment occurred (2–3 h). The cells were then briefly washed, and exposed to glucocorticoid-free or dexamethasone-supplemented medium, as detailed in the text.

Electrophysiology

Membrane currents were recorded (Axopatch 200B amplifier, Axon Digidata 1440A interface, pClamp 10 software, Axon Instruments, Foster City, CA, USA) from cells held under voltage clamp in the perforated patch recording configuration (Horn and Marty, 1988) using amphotericin to gain electrical access to the cell interior (Clunes *et al.*, 2004). The pipette filling solution contained (in mM) NaCl, 10; KCl, 18; K gluconate, 92; $MgCl_2$, 0.5; EGTA, 1; HEPES 10; its pH was adjusted to 7.2 with KOH, which brought $[K^+]$ to 113.3 mM. The standard bath solution contained (in mM) NaCl 140; KCl 4.5; $MgCl_2$ 1; $CaCl_2$ 2.5; HEPES, 10; glucose 5; its pH was adjusted to 7.4 with NaOH, which brought $[Na^+]$ to 144.4 mM. Experiments were undertaken at room temperature (~22°C) and the equilibrium potential for Na^+ (E_{Na}) and K^+ (E_K) were therefore 67.8 mV and –81.9 mV respectively. Recordings were initiated once the access resistance (R_a) had fallen to a stable value below 50 MΩ (10–15 min). R_a and input capacitance (C_m) were monitored throughout each

experiment and all presented data are from preparations in which these parameters remained stable. We achieved 40–50% compensation of the voltage drop across R_a, and because the lowest value of membrane resistance was ~0.5 GΩ, the residual error in the holding potential (V_{Hold}) was always <~4%; this was assumed to be negligible. In all experiments, the membrane currents evoked by ramp changes in V_{Hold} (−113 mV to +87 mV, 2 s) were initially recorded during exposure to the standard bath solution and the measurement then repeated after 20–30-s exposure to 10 μM amiloride. The amiloride-sensitive component of the total membrane current (I_{Amil}) was isolated by digitally subtracting the current that persisted in the presence of amiloride from the corresponding record of control current. Previous work from this laboratory has shown that selective Na^+ currents can only be recorded from H441 cells growing in contact with each other (Brown *et al.*, 2008) and the electrophysiological data included in the present study are therefore derived entirely from cells growing in groups of three to six cells. The values of C_m measured under these conditions ranged from 20–100 pF and, because the value of C_m measured in an initial study of cells growing singly was 28.8 pF ± 5.2 pF (n = 10); this variability indicates that neighbouring cells can be electrically coupled to each other. There was, however, no obvious relationship between the magnitude of C_m and the number of cells in each group. Indeed, in many instances, we recorded low values of C_m from cells that were clearly in contact with their neighbours, and the extent to which the cells become electrically coupled to their neighbours therefore appears to be variable. Thus the currents reported in the present study flow across the membranes of one to five cells and larger values of C_m were associated with larger currents. To ensure that this variability did not contribute to the variability in the presented data, the magnitudes of all recorded currents have been normalized to C_m (i.e. expressed as pA/pF). For presentation, such data were scaled to the mean value of C_m associated a single cell, so that the presented data are shown as pA per average-sized cell. All potentials are corrected for the liquid junction potntial (−13 mV) between the bath and pipette solutions and cited values of membrane potential (V_m) were inferred from the value of V_{Hold} at which I_m was zero. Data are presented as mean ± SEM and values of n denote the number of recordings made from different cells; all protocols were repeated in cells from at least three different passage numbers.

Phosphorylation of endogenous proteins

Cells on six-well plates were washed with ice-cold PBS and scraped into ice-cold lysis buffer containing protease and phosphatase inhibitors (1% Triton; 50 mM Tris – HCl, pH 7.5; 1 mM EGTA; 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). Lysates were then transferred to Eppendorf tubes, ultrasonicated to ensure complete cellular disruption and their protein contents determined using Bradford reagent. Aliquots of extracted protein were reduced and denatured by heating (95°C, 5 min) in the presence of 5% β-mercapto-ethanol and 2% SDS and fractionated on SDS-polyacrylamide gels. Fractionated proteins were then transferred to Hybond-P membranes (GE Healthcare, Buckinghamshire, UK) that were probed using antibodies against the $Thr^{346/356/366}$-phosphorylated and total forms of the protein encoded by the N-myc dowstream regulated gene 1 (NDRG1); the Ser^{473}-phosphorylated, Thr^{308}-phosphorylated and total forms of protein kinase B (PKB); the Thr^{389}-phosphorylated and total forms of 70 kDa ribosomal S6 kinase (P70-S6K), and the Ser^{240}-phosphorylated and total forms of the 40 kDa proline-rich substrate of Akt/PKB (PRAS40). The antibodies against NDRG1 and PRAS-40 were generously made available by Prof. Sir Philip Cohen (MRC-Protein Phosphorylation Unit, University of Dundee) and the antibodies against PKB and P70-S6K were from Cell Signalling (Hertfordshire, UK). Molecular weights of identified bands were estimated by comparing their electrophoretic mobilities with those of a series of protein standards.

Expression of ENaC subunits

Cells on six-well plates were placed on ice, extensively washed with ice-cold PBS and then exposed (1 h at 4°C with gentle agitation) to 10 mM sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3′dithioproprionate (EZ-Link Sulfo-NHS-SS-Biotin, Pierce, Fisher Scientific, West Sussex, UK), a reagent that covalently modifies proteins by attaching a cleavable biotin moiety to amine groups in N-terminal amino acid residues and in lysine side chains. The biotinylation reaction was terminated with excess substrate (ice-cold 100 mM glycine) and the cells then lysed as described above. All lysates were centrifuged at 14 000 x g for 30 min to precipitate cell debris/insoluble proteins, and the protein content of the cleared samples determined using Bradford reagent (Bio-Rad, Hemel Hempstead, UK). Protein that had simply been extracted from the cells in this way is referred to as 'total cellular protein'. In an initial series of experiments, aliquots (500 μg) of total protein were mixed with streptavidin-coated agarose beads (Pierce, Fisher Scientific) and allowed to equilibrate for 60 min at room temperature with continual agitation. Streptavidin binds biotin with high affinity and proteins that have been modified by exposure to the sulpho-NHS-SS-biotin reagent will thus bind to these beads. The beads were washed extensively to remove unbound proteins and then heated to 95°C under strongly reducing/denaturing conditions (5% β-mercapto-ethanol/2% SDS) in order to cleave the disulphide bonds in the biotin moiety and release bound proteins into solution. The protein purified in this way is referred to as 'biotinylated protein'. Experiments (n = 8) in which the mass of biotinylated protein that could be purified using a relatively large volume (500 μL) of streptavidin-bead slurry was assayed using a protein assay compatible with reducing/denaturing agents (RC–DC reagent, Bio-Rad, Hemel Hempstead, UK), showed that 48.0 ± 0.7 μg of protein was recovered from an initial 500 μg aliquot. Because the protein-binding capacity of the streptavidin-coated beads is 1–3 $mg \cdot mL^{-1}$ per slurry, all subsequent experiments used 150 μL of slurry to isolate the biotinylated protein from 500 μg of total protein. Under these conditions, there is an approximate threefold excess binding capacity and the smaller bead volume allows the biotinylated proteins to be released directly into 50 μL of the sample buffer used for SDS-PAGE. Western analysis showed that β-actin was readily detectable in 1 μg aliquots of total protein and, although β-actin could also be detected in 20 μg aliquots of biotiny-

lated protein, it was necessary to overexpose the blots in order to detect this protein. Because β-actin is a cytoskeletal protein that is confined to the inner leaflet of the plasma membrane, this protein would be inaccessible to biotinylation reagents in the extracellular fluid. The presence of β-actin in the biotinylated protein fraction thus shows that this protein pool does contain some intracellular proteins. However, densitometric analysis showed that the abundance of β-actin in a 20 μg aliquot of biotinylated protein was ~30% of the abundance measured in 1 μg of total protein. This finding indicates that the surface biotinylation/streptavidin binding protocol allows surface-exposed proteins to be isolated with 98–99% purity, and this protein fraction is therefore referred to as the 'surface protein'. In all subsequent experiments, aliquots of total protein (40 μg) and of the surface protein purified from 500 μg of total protein were subject to Western blot analysis using antibodies against α-, β- and γ-ENaC, and β-actin. The antibody against α-ENaC was a gift from Cystic Fibrosis Center, University of North Carolina (Chapel Hill, NC, USA); the antibody against β-ENaC was from Santa Cruz Biotechnology (Heidelberg, Germany), while the γ-ENaC and β-actin antibodies were from Sigma Chemical Co.

Data analysis and experimental design

The results of experiments that simply explored the effects of exposing cells to a single test substance were analysed using Student's *t*-test, while the data from experiments that followed more complex protocols were analysed using one-way ANOVA with a Bonferroni *post hoc* test. Experiments were undertaken using strictly paired experimental designs in which the control and experimental cells were harvested from the same culture flasks and cultured in parallel. Care was taken to ensure that the control and experimental cells were subject to an identical series of solution changes, and none of the effects reported here can therefore be attributed to variations in physical disturbance. Protein samples from control and experimental cells were extracted and processed in parallel using identical reagents; fractionated on the same gels, and Western blots probed using identical antibodies and other reagents. For quantitative analysis, a computer scanner was used to produce image files of each blot, and a standard software package (ImageJ) was then used to quantify the optical density of the bands corresponding to control/experimental protein. Background subtraction was achieved by subtracting the optical density measured for an identically sized area of clear gel adjacent to the region of interest. In order to present the pooled results from a series of experiments, we first calculated the mean optical density for the control samples. All individual data points were then normalized to this mean value, and all such data are therefore presented as relative abundances. All presented images were processed digitally and, although minor adjustments were made to contrast/brightness, these changes were applied to the entire image. No specific feature visible in any image has therefore been enhanced, obscured, moved or introduced.

Materials

Amiloride, dexamethasone and rapamycin were from Sigma Chemical Co., Poole, Dorset; PI103 from Merck KGaA, Darmstadt, Germany and GSK650394 from TOCRIS Bioscience, Bristol, UK. TORIN1 was a kind gift from Prof David Sabatini (Whitehead Institute for Biomedical Research, Cambridge, Mass., USA).

Results

Dexamethasone-induced activation of ENaC

Analysis of the total membrane currents recorded from glucocorticoid-deprived cells showed that the relationship between I_m and V_{Hold} was essentially linear at physiologically relevant values of V_{Hold} but displayed outward rectification at depolarized potentials (Figure 1A); V_m was normally ~−45 mV (Figure 1A,C). Amiloride (10 μM) had no effect upon the total membrane current recorded from such cells and therefore caused no change in V_m (Figure 1A,C), and further analysis of these data thus showed that I_{Amil} (see Methods) was negligible (Figure 1D). Studies of dexamethasone-treated (0.2 μM, ~24 h) cells, on the other hand, revealed relatively depolarized values of V_m (Figure 1B,C) and these hormone-treated cells displayed hyperpolarizing responses to 10 μM amiloride (ΔV_m ~−40 mV, Figure 1B); further analysis of these data revealed an amiloride-sensitive component to the total membrane current that reversed at a potential (~65 mV) close to E_{Na} (Figure 1D); this current (I_{Amil}) is therefore Na^+ selective.

Kinase inhibitors suppress the phosphorylation of endogenous protein substrates

Analysis of protein extracted from dexamethasone-treated (0.2 μM, 24 h) cells revealed clearly detectible levels of Ser^{473}-phosphorylated (Figure 2A,B) and Thr^{308}-phosphorylated (Figure 2C,D) PKB and, because these residues are physiological substrates for TORC2 and PDK1, respectively (Biondi *et al.*, 2001; Bayascas and Alessi, 2005), this result shows that these PI3K-dependent protein kinases are active under these conditions. Such analyses also revealed phosphorylation of NDRG1-$Thr^{346/356/366}$ (Figure 2E,F), PRAS40-Ser^{240} (not shown, $n = 3$) and P70-S6K-Thr^{389} (not shown, $n = 3$) and, since these residues are physiological substrates for SGK1, PKB and the target of rapamycin signalling complex 1 (TORC1), respectively (Kovacina *et al.*, 2003; Murray *et al.*, 2004; 2005; Proud, 2007), it is clear that these PI3K-dependent protein kinases are also active in dexamethasone-treated cells. PI103 (0.5 μM, 3 h) appeared to cause a marked reduction in the cellular abundance of Ser^{473}-phosphorylated (Figure 2A) and Thr^{308}-phosphorylated (Figure 2C) PKB, and densitometric/statistical analysis of the data from the entire series of such experiments confirmed that these effects were statistically significant (Figure 2B,D). Moreover, PI103 had no effect upon the overall PKB expression level (Figure 2B,D) and therefore causes virtually complete inactivation of TORC2 and PDK1. PI103 also caused a marked fall in the abundance of $Thr^{346/356/366}$-phosphorylated NDRG1 without altering the overall NDRG1 expression level (Figure 2E,F), and it is therefore clear that this substance also inactivates SGK1. PI103 (0.5 μM, 3 h) similarly suppressed the phosphorylation of PRAS40-Ser^{240} ($n = 3$, not shown) demonstrating inactivation of PKB. Rapamycin (0.1 μM, 3 h), a highly selective inhibitor of TORC1

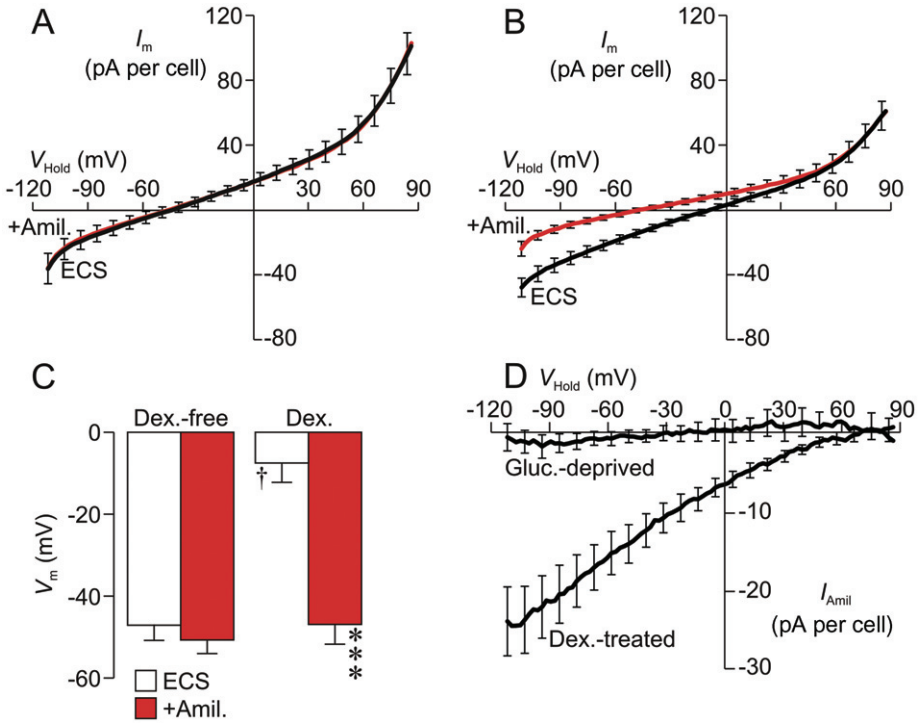

Figure 1

Prolonged (24 h) exposure to dexamethasone activates epithelial Na^+ channels (ENaC). (A) Relationships (n = 12, C_m = 45.7 ± 5.2 pF; R_a = 22.2 ± 4.1 MΩ) between total membrane current (I_m) and holding potential (V_{Hold}) quantified in glucocorticoid-deprived cells during exposure to the standard extracellular solution (ECS) and after 20–30 s exposure to 10 μM amiloride (+Amil.). (B) Equivalent data from dexamethasone-treated (0.2 μM, ~24 h) cells (n = 9, C_m = 47.6 ± 8.0 pF; R_a = 28.9 ± 3.1 MΩ). (C) Values of membrane potential (V_m) derived by analysis of the data in A and B (means ± SEM). ***P < 0.001, significant effects of amiloride, Student's paired t–test. †P < 0.05, significant effect of dexamethasone, one-way ANOVA with Bonferroni *post hoc* test. (D). The amiloride-sensitive component of the total membrane current (I_{Amil}) was isolated by further analysis of the data in A (Gluc.-deprived) and B (Dex.-treated) and has been plotted against V_{Hold}.

(Bain *et al.*, 2007) did not alter the phosphorylation status of PKB-Ser^{473} (Figure 2A,B), PKB-Thr^{308} (Figure 2C,D) or NDRG1-Thr$^{346/356/366}$ (Figure 2E,F) but did suppress the phosphorylation of P70-S6K-Thr^{389} (not shown, n = 3), a TORC1 substrate (Proud, 2007) indicating essentially inactivation of this protein kinase. GSK650394 (10 μM, 3 h) had no effect upon the phosphorylation of PKB-Ser^{473} (Figure 2A,B), PKB-Thr^{308} (Figure 2C,D) or PRAS40-Ser^{240} (n = 3, not shown) but did suppress the phosphorylation of NDRG1-Thr$^{346/356/366}$ (Figure 2E,F) indicating selective inactivation of SGK1. TORIN1, a recently developed inhibitor of TORC1 and TORC2 (Thoreen *et al.*, 2009), caused a concentration-dependent fall in the abundance of Ser^{473}-phosphorylated PKB that occurred without influencing the overall PKB expression level, and this effect was complete at 0.1 μM (Figure 3A,B). While high concentration of TORIN1 also significantly suppressed the phosphorylation of PKB-Thr^{308}, this effect (~50% inhibition, Figure 3C,D) was smaller (P < 0.001, Student's t-test) than the effect on PKB-Ser^{473} (~90% inhibition, Figure 3A,B), and statistical analysis of these data confirmed (P < 0.05) that TORIN1 inhibited the phosphorylation of PKB-Ser^{473} more effectively than it suppressed the phosphorylation of PKB-Thr^{308}. TORIN1 (0.1 μM, 3 h) also virtually abolished the phosphorylation of NDRG1-Thr$^{346/356/366}$ (Figure 3E,F) and, because rapamycin had no such effect (Figure 2E,F), this apparent inactivation of SGK1 must reflect inhibition of TORC2.

Dexamethasone-induced Na^+ currents are dependent on the PI3K–TORC2–SGK1 pathway

In a further series of electrophysiological studies, total membrane currents were again recorded from cells that had been exposed to 0.2 μM dexamethasone for ~24 h (n = 5). Analysis of these data confirmed (see earlier) that such dexamethasone-treated cells display depolarized values of V_m (0.4 ± 8.1 mV), and analysis of the total membrane currents recorded after 10–30 s exposure to 10 μM amiloride showed that V_m had fallen to –67.1 ± 9.2 mV (P < 0.001). These data thus confirm that this ENaC blocker hyperpolarizes dexamethasone-stimulated cells and further analysis (Figure 4A) revealed an amiloride-sensitive component to the total membrane current essentially identical to that described earlier; the magnitude of the amiloride-sensitive current flowing at E_K ($I_{Amil(-82\ mV)}$) was approximately –50 pA per cell (Figure 4E). Parallel experiments (n = 6) in which total membrane currents were recorded from dexamethasone-treated (0.2 μM, ~24 h) cells that had been exposed to 0.5 μM PI103 for 3–4 h showed that this inhibitor of PI3K hyperpolarized V_m to –51.0 ± 6.0 mV (P < 0.001), and amiloride had no effect

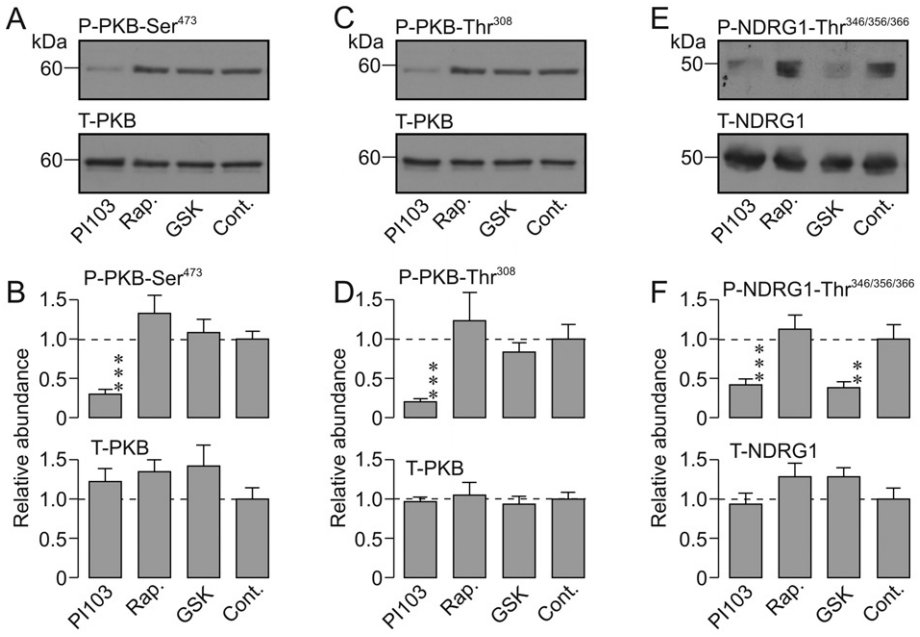

Figure 2

Phosphorylation status of endogenous kinase substrates. Control (Cont) cells were maintained in dexamethasone-containing (0.2 μM) medium for 24 h before being used in experiments while inhibitor-treated cells were exposed to 0.5 μM PI103 (PI103, $n = 11$), 0.1 μM rapamycin (Rap., $n = 8$) or 10 μM GSK650394 (GSK, $n = 4$) for the final 3 h of this incubation period. Aliquots of extracted protein (40 μg) were then subject to Western analysis using antibodies against (A, B) Ser^{473}-phosphorylated/total protein kinase B (P-PKB-Ser^{473}/T-PKB); (C, D) Thr^{308}-phosphorylated/total PKB (P-PKB-Thr^{308}/T-PKB), and (E, F) $Thr^{346/356/366}$-phosphorylated/total n-myc downstream regulated gene 1 protein (P-NDRG1-$Thr^{346/356/366}$/T-NDRG1). A, C and E each show representative blots from single experiments while B, D and F present the pooled data obtained by densitometric analysis of the entire series of experiments (means ± SEM). ***$P < 0.01$; **$P < 0.02$, significantly different from control, Student's paired *t*-test.

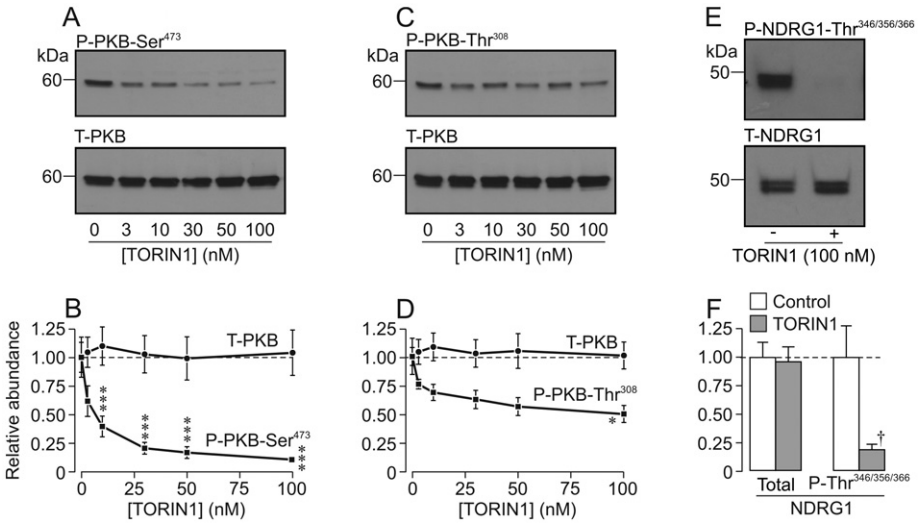

Figure 3

Effects of TORIN1. Dexamethasone-treated (0.2 μM, 24 h) cells were either maintained under control conditions or exposed to TORIN1 (3–100 nM) for the final 3 h of this incubation period. Aliquots of protein extracted from control and TORIN1-treated cells ($n = 5$ for each) were then analysed by Western blotting using antibodies against Ser^{473}-phosphorylated/total protein kinase B (P-PKB-Ser^{473}/T-PKB, A, B), Thr^{308}-phosphorylated/total PKB (P-PKB-Thr^{308}/T-PKB, C, D) and $Thr^{346/356/366}$-phosphorylated/total protein encoded by n-myc downstream regulated gene 1 (P-NDRG1-$Thr^{346/356/366}$/T-NDRG1, E, F). A, C and E show representative blots from single experiments while B, D and F show pooled data derived from densitometric analysis of the entire series of experiments (mean ± SEM). ***$P < 0.01$, **$P < 0.05$, one-way ANOVA with Bonferroni *post hoc* test.; †$P < 0.05$, Student's paired *t*–test; significant effects of TORIN1.

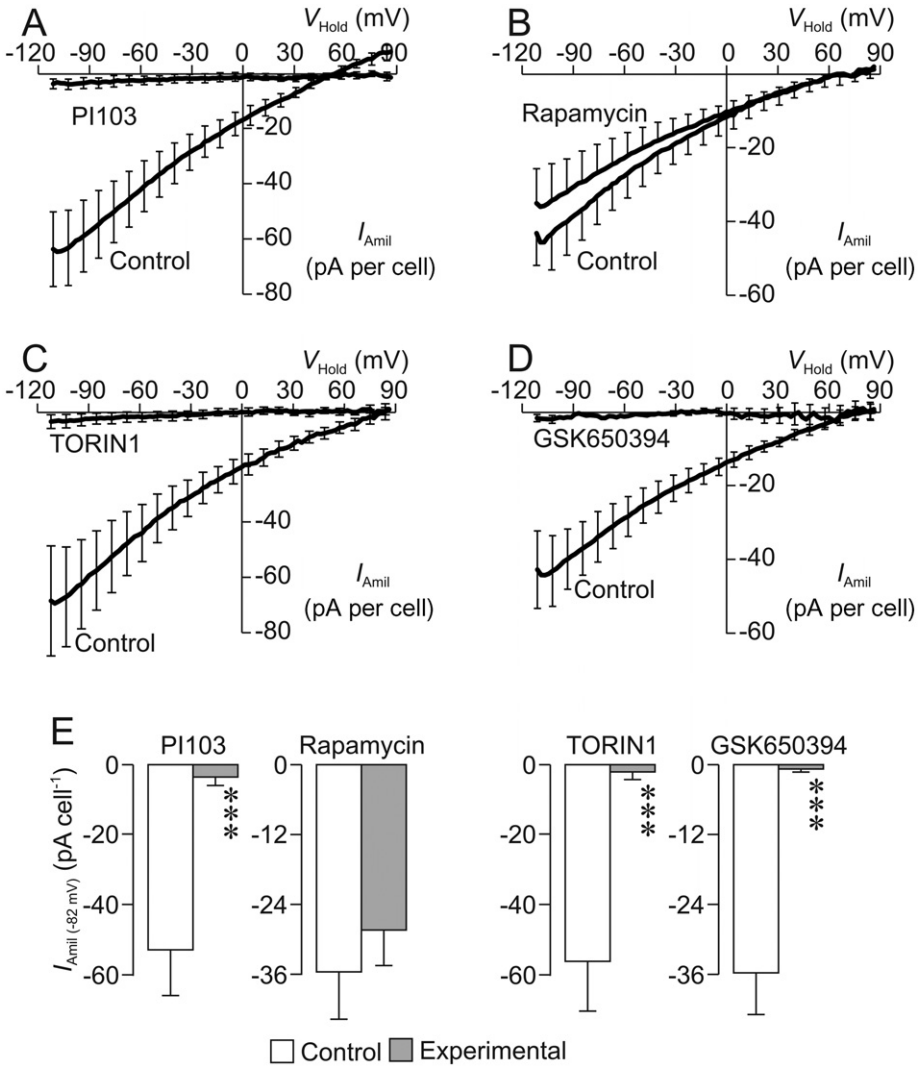

Figure 4

Dexamethasone-induced Na^+ currents – effects of kinase inhibitors. Total membrane currents (I_m) were recorded both under standard conditions and after 10–30 s exposure to 10 μM amiloride. The amiloride-sensitive component of the total membrane current (I_{Amil}) was then isolated by digitally subtracting the current that persisted in the presence of amiloride from the corresponding record of control current, and plotted (mean ± SEM) against V_{Hold}. (A) Data from cells exposed to 0.2 μM dexamethasone for ~24 h under control conditions, and from cells that had also been exposed to 0.5 μM PI103 for the final 3–4 h of this incubation period (control: n = 5, C_m = 55.3 ± 10.8 pF; R_a = 27.5 ± 11.9 MΩ; PI103-treated: n = 6, C_m = 46.2 ± 9.3 pF; R_a = 32.8 ± 8.4 MΩ). (B) Data from experiments that used an identical protocol to explore the effects of 0.1 μM rapamycin (3–4 h) upon I_{Amil} in dexamethasone-treated (0.2 μM, ~24 h) cells (control: n = 4, C_m = 67.4 ± 13.4 pF; R_a = 27.8 ± 10.8 MΩ; rapamycin-treated: n = 5, C_m = 79.3 ± 19.3 pF; R_a = 30.7 ± 4.1 MΩ). (C) Analogous data showing the effects of 0.1 μM TORIN1 (control: n = 4, C_m = 39.9 ± 10.1 pF; R_a = 20.1 ± 3.9 MΩ; TORIN1-treated: n = 4, C_m = 40.5 ± 5.8 pF; R_a = 33.1 ± 6.9 MΩ). (D) Analogous data showing the effects of 10 μM GSK650394 (control: n = 5, C_m = 69.7 ± 11.7 pF; R_a = 39.4 ± 10.1 MΩ; GSK650394-treated: n = 4, C_m = 35.4 ± 2.9 pF; R_a = 39.8 ± 11.8 MΩ). (E) Pooled data showing the amiloride-sensitive currents flowing at –82 mV ($I_{Amil\ (-82\ mV)}$, mean ± SEM) quantified in control (i.e. 0.2 μM dexamethasone, ~24 h) and inhibitor-treated (3–4 h) cells. ***P < 0.001, significantly different from appropriate control, Student's t-test.

upon the currents recorded under these conditions and thus caused no change in V_m (ΔV_m = –6.3 ± 3.6 mV). Further analysis showed that PI103 abolished the amiloride-sensitive Na^+-current that is normally seen in dexamethasone-treated cells (Figure 4A,E). Exposing dexamethasone-treated cells to rapamycin (0.1 μM, 3–4 h), on the other hand, did not alter V_m (control: –3.9 ± 5.3 mV, n = 4; rapamycin: –9.8 ± 4.2 mV, n = 4); did not modify the response to amiloride (control: ΔV_m = –62.9 ± 10.2 mV; rapamycin: ΔV_m = –36.7 ± 17.4 mV), and did not suppress the amiloride-sensitive Na^+ current (Figure 4B,E). TORIN1 (0.1 μM, 3–4 h) mimicked the effects of PI103 by hyperpolarizing V_m (control: 8.3 ± 8.7 mV, n = 4; TORIN1: –58.5 ± 9.0 mV, n = 4, P < 0.05), abolishing the hyperpolarizing response to amiloride (control: ΔV_m = –67.6 ± 19.6 mV; TORIN1: ΔV_m = –8.3 ± 7.8 mV, P < 0.05) and essentially abolishing dexamethasone-induced, amiloride-sensitive Na^+ current (Figure 4C,E). Similarly, GSK650394 (10 μM, 3–4 h) also hyperpolarized V_m (control: –10.3 ±

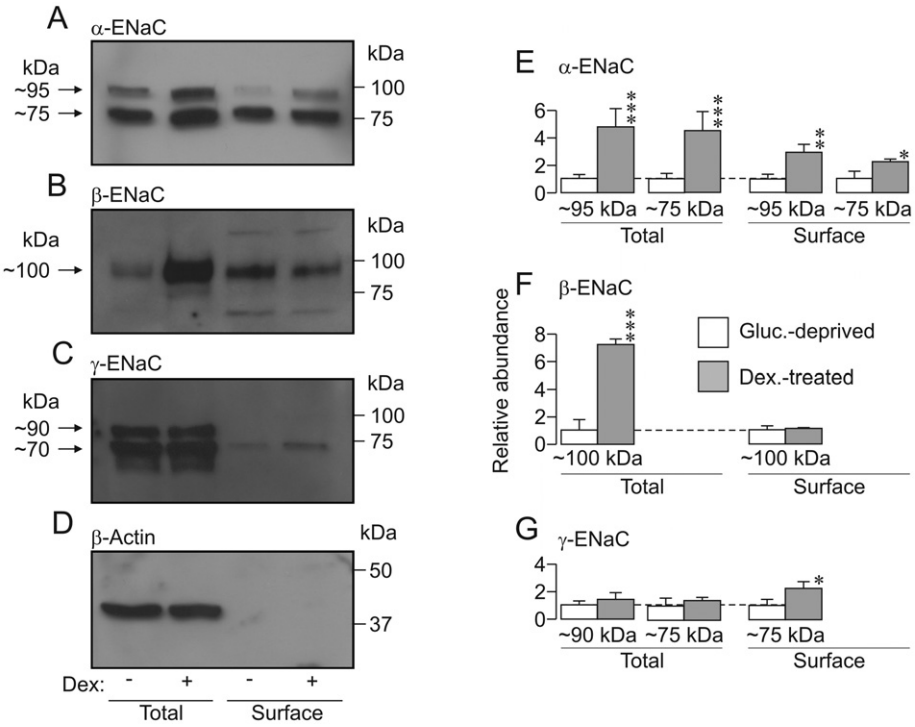

Figure 5

Effects of prolonged (~24 h) exposure to 0.2 μM dexamethasone upon the expression of epithelial Na^+ channel α, β and γ subunits (α-, β- and γ-ENaC). (A) Western blots showing the abundance of α-ENaC in aliquots of total (40 μg) and surface-exposed (purified from 500 μg of total protein) protein derived from glucocorticoid-deprived and dexamethasone-treated (0.2 μM, ~24 h) cells. Analogous data for β- (B), γ-ENaC (C) and *β*-actin (D) are also presented. (E–G) Densitometric analyses showing the pooled results from five independent experiments. *$P < 0.05$; **$P < 0.001$; ***$P < 0.005$, significant effects of dexamethasone, Student's paired *t*-test.

6.2 mV, $n = 5$; GSK650394: –38.0 ± 3.1 mV, $n = 4$, $P < 0.001$); abolished the response to amiloride (control: $\Delta V_m = -55.6 \pm 12.2$ mV; GSK650394: $\Delta V_m = -1.6 \pm 1.7$ mV, $P < 0.001$) and abolished the dexamethasone-induced Na^+ current (Figure 4D,E).

Dexamethasone-induced ENaC expression

The α-ENaC antibody consistently detected two bands (~95 and ~75 kDa) in the total and the surface protein pools (see Methods) and a direct comparison of protein extracted from glucocorticoid-deprived and dexamethasone-stimulated cells (0.2 μM, 24 h) showed that this synthetic glucocorticoid increased the overall abundance of both forms of α-ENaC (Figure 5A,E). Dexamethasone also increased the surface abundance of both forms of α-ENaC, and these changes were similar in magnitude to the effects on overall expression (Figure 5A,E). The antibody against β-ENaC revealed a single band at ~100 kDa that was present in both the total and the surface protein pools and, although dexamethasone (0.2 μM, 24 h) significantly increased the overall abundance of this protein, this synthetic glucocorticoid had no effect upon the surface abundance of β-ENaC (Figure 5B,F). The γ-ENaC antibody identified two bands (~90 and ~75 kDa) in total protein but only the ~75 kDa form of γ-ENaC was present at the cell surface (Figure 5C,G). Although dexamethasone (0.2 μM, 24 h) had no discernible effect upon the pattern of γ-ENaC expression, it did cause a small but significant increase in the surface abundance of the 75 kDa form of γ-ENaC (Figure 5C,G).

Effects of kinase inhibitors on the expression of ENaC subunits in dexamethasone-stimulated cells

Figures 6–9 show the results of experiments that used a strictly paired experimental protocol to study the effects of protein kinase inhibitors (3 h) upon the pattern of α-, β- and γ-ENaC expression in dexamethasone-stimulated (0.2 μM, 24 h) cells. The control data in these figures confirm the pattern of expression described earlier. PI103 (0.5 μM, Figure 6), rapamycin (0.1 μM, Figure 7), TORIN1 (0.1 μM, Figure 8) and GSK650394 (10 μM, Figure 9) all reduced the overall abundance of the 95 kDa form of α-ENaC, and these effects were accompanied by corresponding reductions in the surface abundance of this channel subunit. However, these substances did not alter the abundance of the 75 kDa form of α-ENaC in the total or the surface-exposed protein pools (Figures 6–9). The kinase inhibitors also had no effect upon the abundance of β-ENaC in either the total or the surface-exposed protein pools and also failed to alter the overall abundance of γ-ENaC (Figures 6–9). However, PI103 (Figure 6), TORIN1 (Figure 8) and GSK650394 (Figure 9) all reduced the amount of the 75 kDa form of γ-ENaC that was

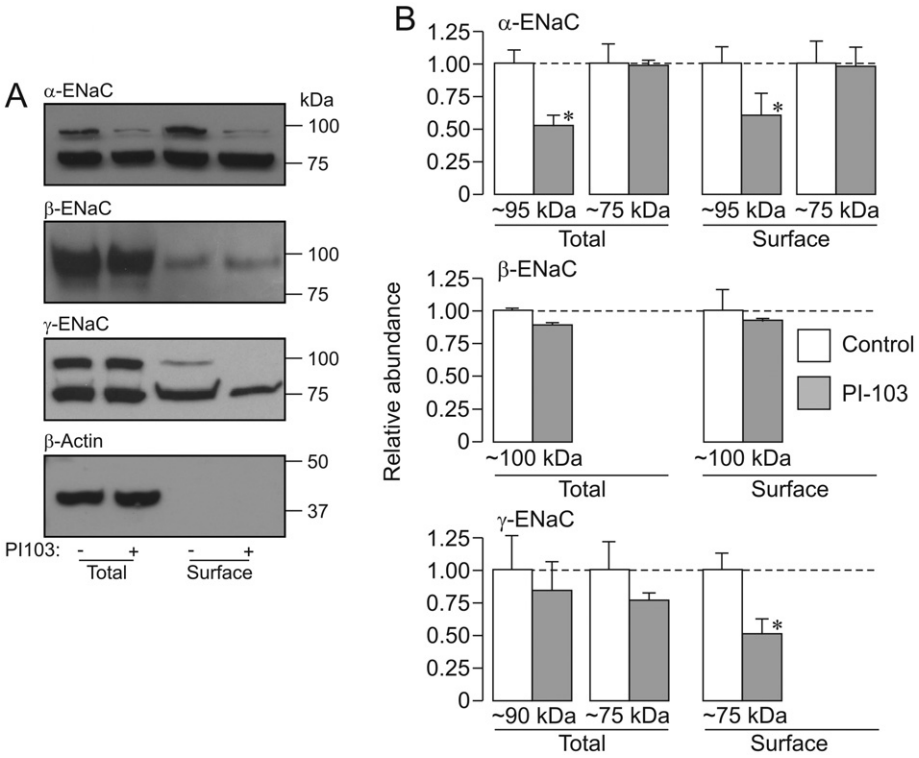

Figure 6

Effects of PI103 upon α-, β- and γ-ENaC expression in dexamethasone-treated cells. Total and surface-exposed proteins were extracted from dexamethasone-treated (0.2 μM, ~24 h) cells that had either been maintained under control conditions or exposed to 0.5 μM PI103 for the final 3 h of the incubation period. The surface-exposed proteins purified from 500 μg aliquots of total protein were fractionated (SDS-PAGE) in parallel with 40 μg aliquots of total protein, and subject to Western analysis using antibodies against α-ENaC, β-ENaC, γ-ENaC and β-actin. (A) Western blot showing data from an individual experiment; bars to the right indicate the approximate position of the appropriate molecular weight markers. (B) Densitometric analysis showing the pooled data from four independent experiments (mean ± SEM). *$P < 0.05$, significant effects of PI103, Student's paired *t*-test.

present at the cell surface. Rapamycin, on the other hand, did not alter the pattern of γ-ENaC expression (Figure 7).

Dexamethasone-induced activation of SGK1 is transient

The data in Figure 10A confirm (Inglis *et al.*, 2009) that dexamethasone activates SGK1 (i.e. evokes phosphorylation of NDRG1-Thr$^{346/356/366}$). This response became apparent after ~30 min and persisted for at least 6 h (Figure 10A,B). Although SGK1 activity is dependent upon the TORC2-catalysed phosphorylation of SGK1-Ser^{422} (Kobayashi and Cohen, 1999; Park *et al.*, 1999; García-Martínez and Alessi, 2008), these experiments also showed that dexamethasone (0.2 μM, 0–6 h) had no significant effect upon the phosphorylation of PKB-Ser^{473} (Figure 10C,D) an endogenous TORC2 substrate (Sarbassov *et al.*, 2005). It is therefore clear that this synthetic glucocorticoid increases cellular SGK1 activity without activating the PI3K – TORC2 pathway. A further series of experiments confirmed that brief (3 h) exposure to dexamethasone (0.2 μM) activated SGK1 (Figure 10E,F) but not TORC2 (Figure 10G,H), but also showed that prolonged (24 h) exposure to dexamethasone had no effect upon the phosphorylation status of NDRG1-Thr$^{346/356/366}$ (Figure 10E,F) or PKB-Ser^{473} (Figure 10G,H). Although dexamethasone clearly activates SGK1, the present data indicate that SGK1 activity returns to its basal level after 24 h of continuous stimulation.

Brief (3 h) exposure to dexamethasone

As the glucocorticoid-induced activation of SGK1 peaked at ~3 h (Figure 10), we also explored the effects of such relatively brief stimulation with dexamethasone upon the surface abundance of α-, β- and γ-ENaC. Western blot analysis confirmed that β-actin was virtually undetectable (not shown, $n = 4$) in the surface-exposed proteins derived from 500 μg of total protein (see Methods), a result that confirms that the biotinylation/streptavidin-binding protocol allows this protein pool to be isolated with high purity. These studies also confirmed that α-ENaC (~95 kDa and ~75 kDa), β-ENaC (~100 kDa) and γ-ENaC (~75 kDa) are all present in the plasma membranes of glucocorticoid-deprived cells (Figure 11). More importantly, these experiments clearly showed that brief (i.e. 3 h) stimulation with dexamethasone caused an unambiguous increase in the surface abundance of each of these subunits (Figure 11). Moreover, this response was abolished by GSK650394 (10 μM), and it is therefore clear that

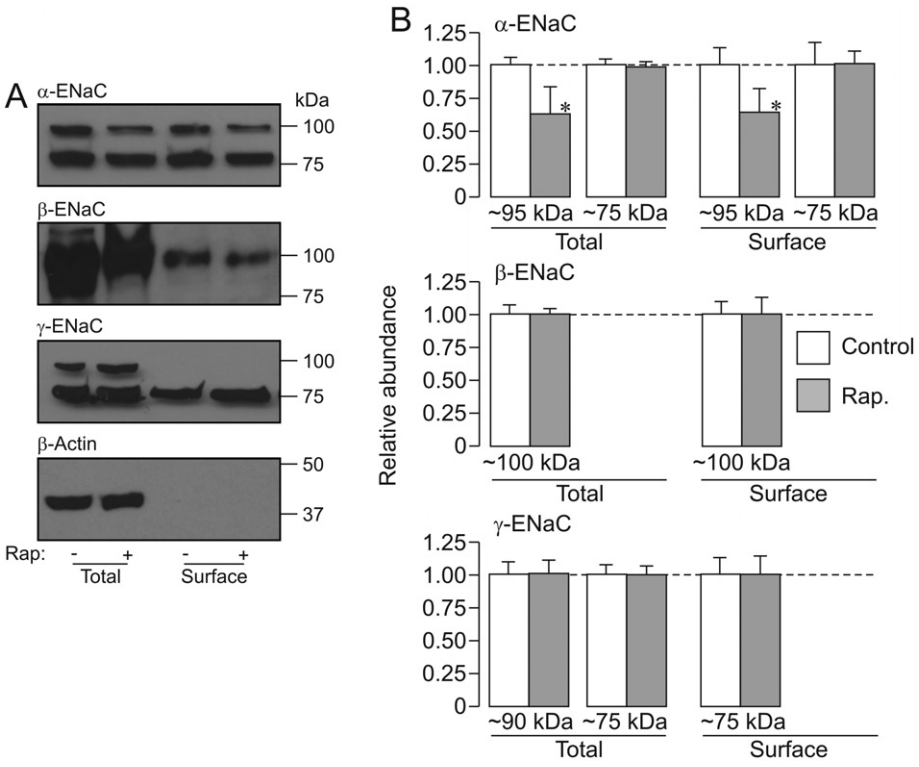

Figure 7

Effects of rapamycin upon α-, β- and γ-ENaC expression in dexamethasone-treated (0.2 μM, ~24 h) cells. Total and surface-exposed proteins were extracted from dexamethasone-treated (0.2 μM, ~24 h) control cells and from dexamethasone-treated cells that had been exposed to 0.1 μM rapamycin for the final 3 h of the incubation period, and analysed as described in Figure 5. (A) Western blot showing data from an individual experiment; bars to the right indicate the approximate position of the appropriate molecular weight markers. (B) Densitometric analysis showing the pooled data from four independent experiments (mean ± SEM). *$P < 0.05$, significant effects of rapamycin, Student's paired *t*-test.

brief (3 h) exposure to dexamethasone induces an SGK1-dependent increase in the surface abundance of α-, β- and γ-ENaC (Figure 11). This observation prompted a further series of electrophysiological studies that used a strictly paired experimental design to compare the effects of brief (3–4 h) and prolonged (~24 h) exposure to dexamethasone (0.2 μM) upon the activity of ENaC. Analysis of the total membrane currents recorded from these cells confirmed that glucocorticoid-deprived cells ($n = 3$) displayed hyperpolarized values of V_m (–64.4 ± 4.2 mV) that were insensitive to 10 μM amiloride (ΔV_m = –0.2 ± 1.5 mV), and I_{Amil} was therefore negligible (Figure 12). Moreover, experiments undertaken using cells that had been exposed to dexamethasone for ~3 h revealed essentially identical values of V_m (–60.8 ± 7.2 mV) and these cells, in common with the glucocorticoid-deprived cells, were insensitive to amiloride ($\Delta V_m = –0.5 \pm 0.7$ mV) and did not display discernible Na^+ currents (Figure 12). However, parallel studies of cells that had been stimulated with dexamethasone for ~24 h revealed depolarized values of V_m (–7.9 ± 7.3 mV) that differed significantly from the values measured in glucocorticoid-deprived cells and in cells that had been exposed to dexamethasone for only 3 h ($P < 0.05$, one-way ANOVA/Bonferroni *post hoc* test). Moreover, these cells displayed hyperpolarizing responses to 10 μM amiloride (ΔV_m = –57.8 ± 0.7 mV, $P < 0.005$, Student's paired *t*-test) and further analysis (Figure 12) revealed amiloride-sensitive Na^+ currents essentially identical to those described earlier.

Discussion

Dexamethasone depolarized H441 cells by activating an endogenous conductance essentially identical to that associated with co-expression of α-, β- and γ-ENaC and this observation confirms (see Lazrak and Matalon, 2003; Clunes *et al.*, 2004; Brown *et al.*, 2008; Althaus *et al.*, 2010) that ENaC activity in these cells is strictly dependent upon glucocorticoid stimulation. Glucocorticoids are physiologically important regulators of pulmonary Na^+ transport and, since these hormones stimulate the removal of fluid from the alveolar space (Matalon and O'Brodovich, 1999; Matthay *et al.*, 2002; Olver *et al.*, 2004), synthetic glucocorticoids are used in the clinical management of conditions such as neonatal respiratory distress syndrome and pulmonary oedema that are characterized by the accumulation of liquid in this region of the lung. Moreover, as glucocorticoids also exert powerful anti-inflammatory actions, glucocorticoid receptor agonists are also used in the treatment of lung diseases such as asthma and chronic obstructive pulmonary disease that are characterized by chronic inflammation (Barnes, 2011). It is therefore

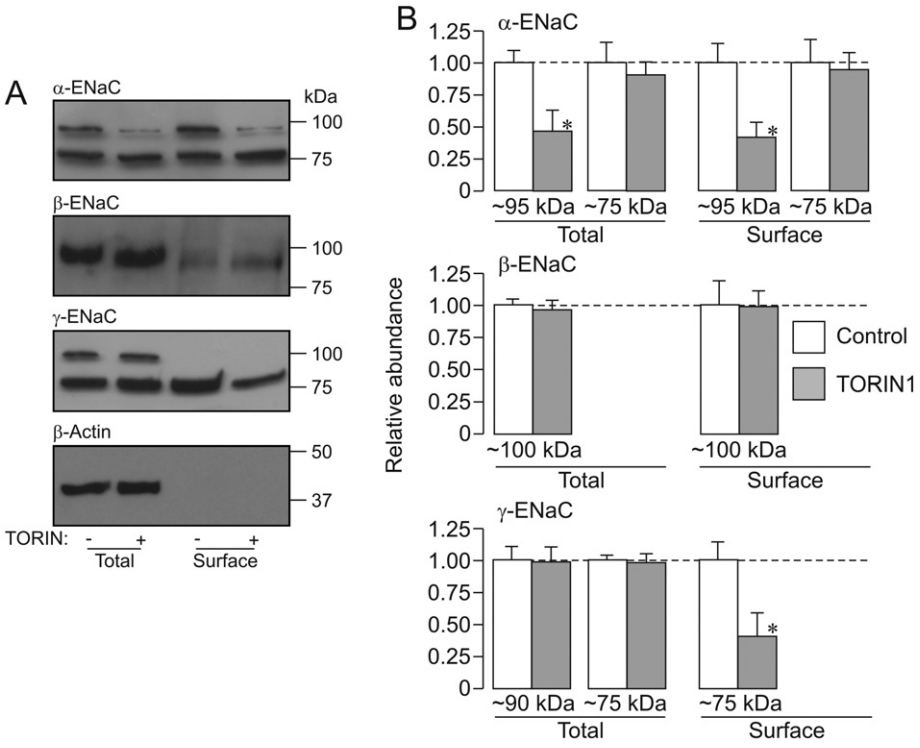

Figure 8

Effects of TORIN1 upon α-, β- and γ-ENaC expression in dexamethasone-treated (0.2 μM, ~24 h) cells. Total and surface-exposed proteins were from dexamethasone-treated (0.2 μM, ~24 h) control cells and from cells exposed to 0.1 μM TORIN1 for the final 3 h of the incubation period were analysed as described in Figure 5 (A) Western blot showing the results of an individual experiment; bars to the right indicate the approximate position of the appropriate molecular weight markers. (B) Densitometric analysis showing the pooled data from four independent experiments (mean ± sSEM). *$P < 0.05$, significant effects of TORIN1, Student's paired *t*-test.

important to understand the mechanisms that allow pulmonary epithelial cells to respond to these hormones and, in order to study the mechanisms that underlie the activation of endogenous ENaC, we have now explored the effects of low MW inhibitors of various kinases upon the dexamethasone-dependent Na^+ currents seen in these cells. However, this approach is complicated by the fact that such compounds almost invariably act upon multiple targets (Bain *et al.*, 2007) and we therefore also used phospho-specific antibodies to monitor the phosphorylation status of endogenous kinase substrates in order to ensure that the tested compounds caused full inhibition of their targets without inactivating other, closely related kinases.

Effects of kinase inhibitors

Analysis of proteins extracted from dexamethasone-stimulated cells showed that PDK1 and TORC2 were both active and, since these kinases depend upon the presence of $PIP_{2/3}$ in the plasma membrane (Biondi *et al.*, 2001; Bayascas and Alessi, 2005; García-Martínez and Alessi, 2008), this result also suggests that PI3K is active under these conditions. These studies revealed the activity of SGK1 and PKB and this was anticipated because these regulatory kinases lie downstream of PI3K – TORC2/PDK1 (Kobayashi and Cohen, 1999; Park *et al.*, 1999; Biondi *et al.*, 2001; Bayascas and Alessi, 2005; Sarbassov *et al.*, 2005; García-Martínez and Alessi, 2008). PI103 caused inactivation of PDK1, TORC2, SGK1 and PKB, and this broad action is consistent with selective inhibition of PI3K since the inactivation of this phospholipid kinase would deplete the membrane of $PIP_{2/3}$ leading to inactivation of PDK1 and TORC2. This effect, in turn, would inactivate SGK1 and PKB (Kobayashi and Cohen, 1999; Park *et al.*, 1999; Bayascas and Alessi, 2005; Sarbassov *et al.*, 2005; García-Martínez and Alessi, 2008). Although PI103 is known to inhibit TORC1 as well as PI3K (Bain *et al.*, 2007; Raynaud *et al.*, 2007), this cannot explain the present data because rapamycin, a highly selective TORC1 inhibitor (Bain *et al.*, 2007), did not mimic the effects of PI103 despite causing essentially complete inactivation of TORC1. Therefore, PI103 provides an effective means of inhibiting PI3K in H441 cells and the fact that PI103, but not rapamycin, abolished the dexamethasone-induced Na^+ current shows that glucocorticoids control ENaC via a PI3K-dependent mechanism. This conclusion is consistent with previous studies (Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; De La Rosa *et al.*, 1999; 2003; Păunescu *et al.*, 2000; Debonneville *et al.*, 2001; Inglis *et al.*, 2009).

In order to study the physiological basis of this requirement for PI3K our subsequent experiments explored the effects of TORIN1 a compound that inhibits TORC2, the protein kinase that allows PI3K to control both PKB and SGK1

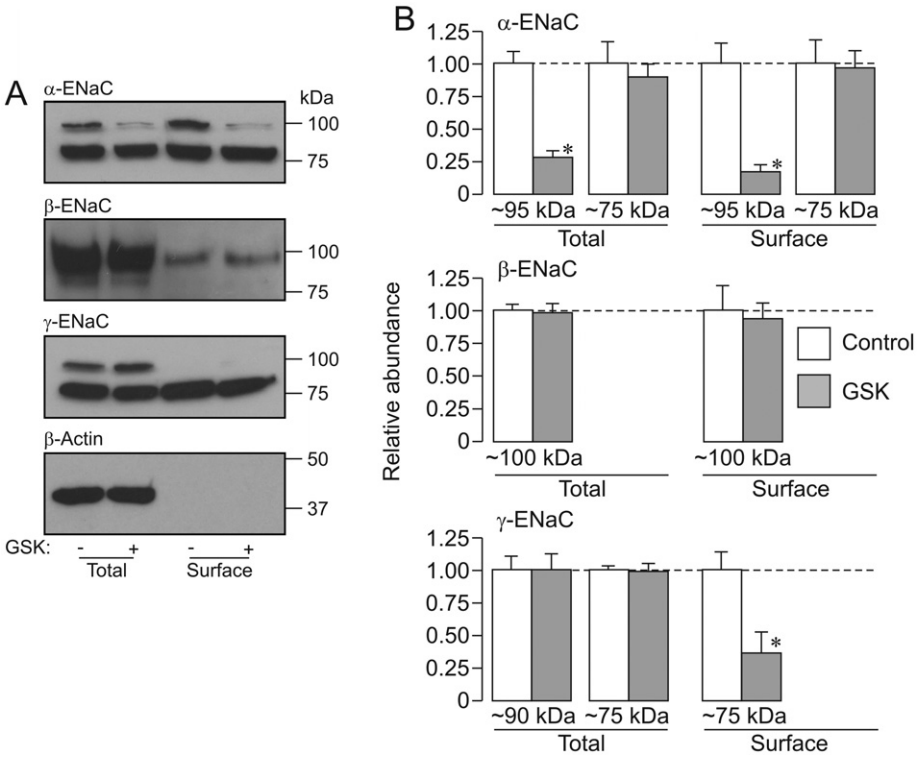

Figure 9

Effects of GSK650394 upon α-, β- and γ-ENaC expression in dexamethasone-treated (0.2 μM, ~24 h) cells. Total and surface-exposed proteins were from dexamethasone-treated (0.2 μM, ~24 h) control cells and from cells exposed to 10 μM GSK650394 for the final 3 h of the incubation period were analysed as described in Figure 5. (A) Western blot showing the results of an individual experiment; bars to the right indicate the approximate position of the appropriate molecular weight markers. (B) Densitometric analysis showing the pooled data from four independent experiments (mean ± SEM). *$P < 0.05$, significant effects of GSK650394, Student's paired *t*-test.

(Sarbassov *et al.*, 2005; García-Martínez and Alessi, 2008; Thoreen *et al.*, 2009). When used at 0.1 μM, TORIN1 virtually abolished the phosphorylation of PKB-Ser^{473}, indicating full inactivation of TORC2, but also suppressed the phosphorylation of PKB-Thr^{308}, a PDK1 substrate (Bayascas and Alessi, 2005). While this effect might indicate direct inhibition of PI3K and/or PDK1, this is unlikely as these kinases are insensitive to TORIN1 at concentrations <1 μM (Thoreen *et al.*, 2009). Moreover, the TORC2-catalysed phosphorylation of PKB-Ser^{473} is known to facilitate the subsequent phosphorylation of PKB-Thr^{308} by PDK1 and this interaction suggests that inhibition of TORC2 may also restrict the phosphorylation of PKB-Thr^{308} (Biondi *et al.*, 2001; Sarbassov *et al.*, 2005). Our data are consistent with earlier results that showed that TORIN1 (0.1 μM) inactivates TORC2 without inhibiting PI3K or PDK1 (Thoreen *et al.*, 2009). The fact that TORIN1 also inactivated SGK1 therefore confirms that TORC2 is a critical part of the mechanism that allows PI3K to activate this protein kinase (Kobayashi and Cohen, 1999; Park *et al.*, 1999; García-Martínez and Alessi, 2008; Lu *et al.*, 2010). TORIN1 abolished glucocorticoid-induced ENaC activity as effectively as PI103 indicating that TORC2 is also important to the hormonal control of these channels (see also Lu *et al.*, 2010; Mansley and Wilson, 2010a). Moreover, the fact that TORIN1 does not inhibit PI3K (Thoreen *et al.*, 2009) predicts that $PIP_{2/3}$ will be present in the membranes of TORIN1-treated cells and this is significant because these phospholipid second messengers can activate ENaC by binding directly to the channel complex (Blazer-Yost *et al.*, 2004; Blazer-Yost and Nofziger, 2005; Ma *et al.*, 2007; Pochynyuk *et al.*, 2008). The fact that TORIN1 inactivates ENaC as effectively as PI103 thus implies that $PIP_{2/3}$ cannot support ENaC activity if TORC2 is inactive and, while this does not exclude a role for $PIP_{2/3}$ in the control of ENaC, this mechanism must be of secondary importance in H441 cells.

GSK650394 inactivated SGK1 without affecting PDK1, TORC2 or PKB and these data confirm that this compound is a selective inhibitor of SGK1 (Sherk *et al.*, 2008; Mansley and Wilson, 2010a). The fact that GSK650394 also abolished dexamethasone-induced ENaC activity therefore shows that SGK1 is critical to the glucocorticoid-dependent control of these channels. It is particularly interesting that GSK650394 did not inhibit PKB since this protein kinase has been shown to activate ENaC expressed heterologously in *Xenopus* oocytes or Fisher rat thyroid cells, and it has been suggested that PKB might be involved in the hormonal control of Na+ transport via ENaC (Lee *et al.*, 2007; Diakov *et al.*, 2010). Although the present data do not exclude this possibility, they show that PKB cannot maintain ENaC activity independently of SGK1 in dexamethasone-stimulated H441 cells.

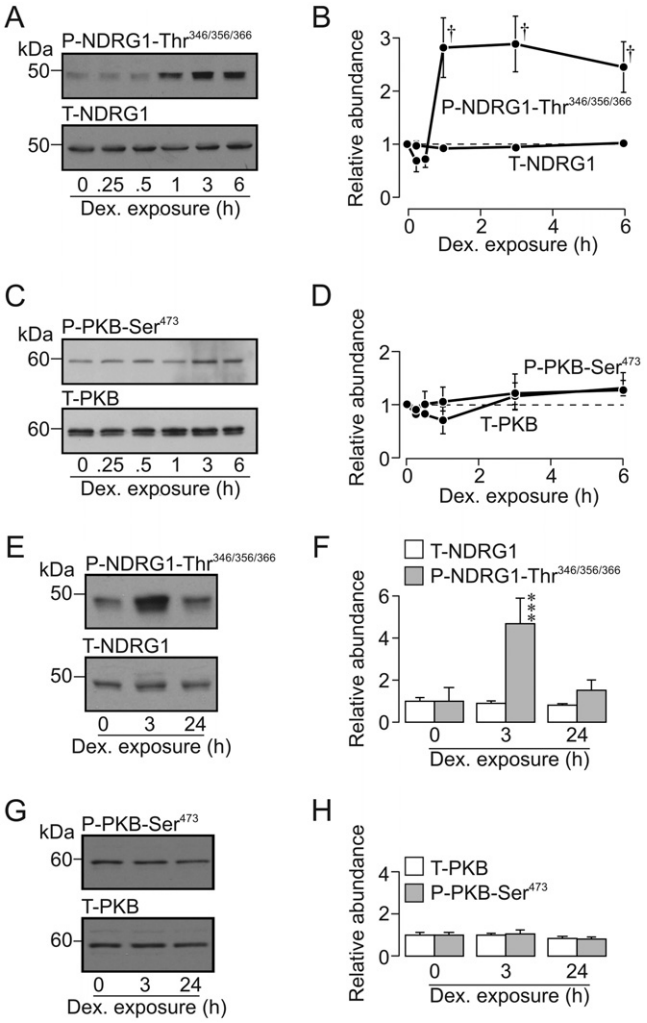

Figure 10

Dexamethasone causes only a transient increase in SGK1 activity. (A) Typical Western blots showing the effects of dexamethasone (0.2 μM, 0–6 h) upon the abundance of $Thr^{346/356/366}$-phosphorylated (upper panel) and total (lower panel) n-myc downstream regulated gene 1 protein (NDRG1). (B) Densitometric analysis showing the pooled data from six such experiments. (C) Typical blots showing the results of experiments that used the same protocol to explore the effects of dexamethasone (0.2 μM, 0–6 h) upon the abundance of Ser^{473}-phosphorylated (upper panel) and total (lower panel) PKB. (D) Densitometric analysis showing the pooled data from six such experiments. (E) Typical Western blots showing the effects of short (3h) and long-term (~24 h) dexamethasone exposure (0.2 μM) upon the abundance of $Thr^{346/356/366}$-phosphorylated and total NDRG1. (F) Densitometric analysis showing the pooled data from five such experiments. (G) Typical Western blots showing the effects of short- (3 h) and long-term (~24 h) dexamethasone stimulation (0.2 μM) upon the abundance of Ser^{473}-phosphorylated (upper panel) and total PKB (lower panel).; essentially identical data were obtained in four independent experiments. (H) Densitometric analysis showing the pooled data from five such experiments. All data are mean ± SEM. †$P < 0.001$, one-way ANOVA with Bonferroni *post hoc* test; ***$P < 0.001$, Student's paired *t*-test, significant effects of dexamethasone.

Glucocorticoid-induced changes in ENaC expression

Detectable levels of α-, β- and γ-ENaC were present in total protein from glucocorticoid-deprived cells and the fact these cells do not display amiloride-sensitive currents (present study, Clunes *et al.*, 2004; Brown *et al.*, 2008; Althaus *et al.*, 2010) cannot, therefore, be attributed to the absence of ENaC. Dexamethasone (24 h) increased the overall expression of α- and β-ENaC, but not γ-ENaC, and this accords with earlier work that has shown that this synthetic glucocorticoid increases the abundance of mRNA encoding α- and β-ENaC in H441 cells (Ramminger *et al.*, 2004; Thomas *et al.*, 2004). Glucocorticoids can therefore control the expression of α- and β-ENaC and, while the mechanism underlying the regulated expression of β-ENaC is unclear, the effect on α-ENaC seems to be mediated by glucocorticoid-response elements in the α-ENaC gene promoter (see Itani *et al.*, 2002; Thomas and Itani, 2004; McTavish *et al.*, 2009).

While the β-ENaC antibody recognized only a single band, α- and γ-ENaC antibodies each identified two bands in total protein derived from glucocorticoid-deprived and dexamethasone-stimulated cells. This observation is consistent with reports that the post translational processing of α- and γ-ENaC, but not β-ENaC, involves cleavage by intracellular and extracellular proteases (see Vallet *et al.*, 1997; Vuagniaux *et al.*, 2002; Hughey *et al.*, 2003; 2004; Caldwell *et al.*, 2004; 2005; Myerburg *et al.*, 2006; 2010; Diakov *et al.*, 2008; Passero *et al.*, 2008; Rossier and Stutts, 2009; Tan *et al.*, 2011). Since these proteases act at different sites, the processing of these channel subunits generates a complex population of proteolytic fragments. The antibodies used in the present study were all directed against N-terminal sequences and molecular weights, which we now report are broadly consistent with the pattern of α- and γ-ENaC cleavage described by Rossier and Stutts, (2009). Although it has been suggested that the cleavage of α- and γ-ENaC is critical for the formation of active channels, we were unable to detect any effect of dexmethasone that was consistent with increased cleavage of these subunits despite the fact that this hormone clearly induced an amiloride-sensitive Na^+ current. However, the data supporting this model are almost entirely derived from studies of heterologously expressed ENaC subunits incorporating synthetic epitopes (e.g. FLAG) that facilitate the identification of cleavage products. While proteases and protease inhibitors clearly modify electrogenic Na^+ transport in absorptive cells and tissues (see, e.g. Myerburg *et al.*, 2006; Lazrak *et al.*, 2009; Tan *et al.*, 2011), few authors have attempted to correlate ENaC activity with changes to the cleavage of endogenous subunits. It is possible that proteolytically cleaved forms of endogenous ENaC are present at a level too low to be detected by conventional methods (Myerburg *et al.*, 2010).

Analysis of surface-exposed proteins showed that α-, β- and γ-ENaC were all present in the membranes of glucocorticoid-deprived and dexamethasone-stimulated cells and, while both forms of α-ENaC were detected, only the lighter form of γ-ENaC was present in this protein pool. Under our experimental conditions the 90 kDa form of γ-ENaC, which may correspond to the full length protein (Rossier and Stutts, 2009), appears to be confined to the

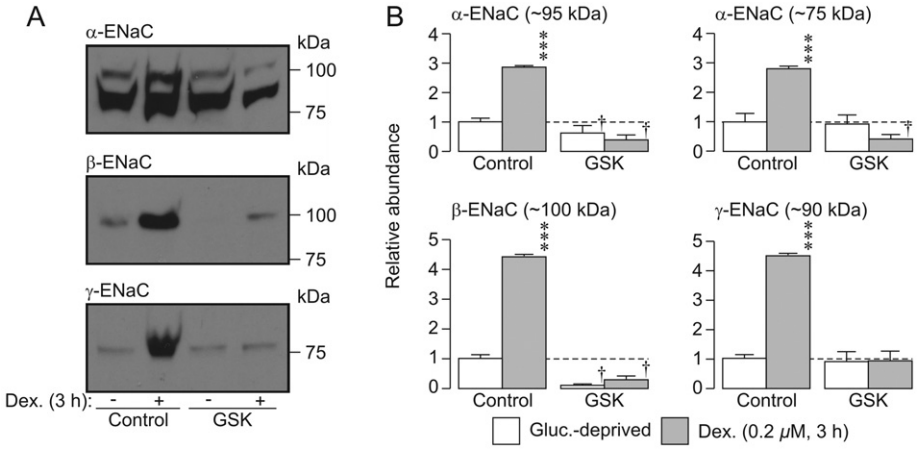

Figure 11

Brief (3 h) stimulation with dexamethasone increases the surface abundance of epithelial Na^+ channel subunits (α-, β- and γ-ENaC). (A) Typical Western blots showing the effects of dexamethasone (0.2 μM, 3 h) upon the surface abundance of epithelial Na^+ channel α, β and γ subunits (α-, β- and γ-ENaC) both under control conditions and in the presence of 10 μM GSK650394 (GSK). (B) Densitometric analysis of the data (mean ± SEM) from four independent experiments. ***$P < 0.001$, significant differences between glucocorticoid-deprived and dexamethasone-stimulated cells,; †$P < 0.05$ significant effects of GSK650394, one-way ANOVA with Bonferroni *post hoc* test.

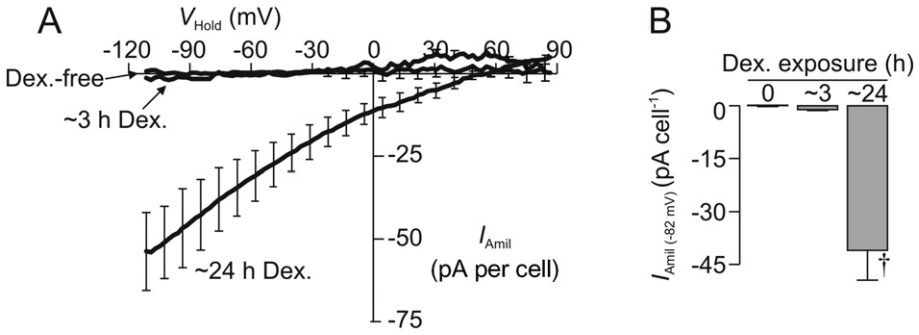

Figure 12

Effects of brief (~3 h) and prolonged (~24 h) stimulation of dexamethasone upon epithelial Na^+ channel activity. (A) Relationships between amiloride-sensitive membrane current (I_{Amil}, mean ± SEM) and holding potential (V_{Hold}) quantified in glucocorticoid-deprived cells (Dex.-free, $n = 3$, $C_m = 47.7 \pm 7.2$ pF; $R_a = 30.7 \pm 9.1$ MΩ) and in cells that had been exposed to 0.2 μM dexamethasone for ~3h (~3 h Dex., $n = 5$, $C_m = 33.6 \pm 2.3$ pF; $R_a = 30.7 \pm 4.8$ MΩ) or ~24 h (~24 h Dex., $n = 5$, $C_m = 48.0 \pm 3.0$pF; $R_a = 19.3 \pm 2.1$ MΩ). (B) Values of $I_{Amil\ (-82\ mV)}$ derived by further analysis of these data. †$P < 0.05$, significantly different from corresponding values in glucocorticoid-free and in ~3 h Dex.-treated cells, one-way ANOVA with Bonferroni *post hoc* test.

intracellular compartment. Although dexamethasone (~24 h) increased the surface expression of α-ENaC, this effect was of similar magnitude to the increase in overall expression. Moreover, prolonged (24 h) exposure to dexamethasone had no effect upon the surface abundance of β-ENaC, and caused only a modest increase in the surface expression of NaC. The present data do not, therefore, support the hypothesis that glucocorticoid-induced (~24 h) epithelial Na^+ currents are due to coordinated increases in the surface abundance of α-, β- and γ-ENaC (Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; De La Rosa *et al.*, 1999; 2003; Păunescu *et al.*, 2000; Debonneville *et al.*, 2001).

Exposing dexamethasone-treated (24 h) cells to PI103, TORIN1 and GSK650394 reduced the surface abundance of γ-ENaC and, because this action was not mimicked by rapamycin, the dexamethasone-induced increase in the surface expression of this subunit seems to be dependent upon the PI3K – TORC2 – SGK1 pathway. This accords well with earlier work (Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; De La Rosa *et al.*, 1999; 2003; Păunescu *et al.*, 2000; Debonneville *et al.*, 2001). However, PI103, TORIN1 or GSK650394 also reduced the amount of the 95 kDa form of α-ENaC that was present in the total and surface-exposed protein pools suggesting that the PI3K–TORC2–SGK1 pathway is involved in the control of α-ENaC synthesis (Boyd & Náray-Fejes-Tóth, 2005; Zhang *et al.*, 2007; Reisenauer *et al.*, 2009). However, most of these compounds also inhibit TORC1 and it is therefore interesting that rapamycin, a selective TORC1 inhibitor (Bain *et al.*, 2007), also reduced the abundance of α-ENaC (95 kDa). It is possible that TORC1 may also contribute to the

control of α-ENaC expression and, in this context, it is relevant that rapamycin has also been shown to disrupt the dexamethasone-induced activation of ENaC in renal epithelia (Mansley and Wilson, 2010a). However, none of the compounds tested in the present study had a discernible effect upon the expression of the 75 kDa form of α-ENaC, and the present data also show that rapamycin-induced inhibition of TORC1 did not suppress ENaC activity in H441 cells. The physiological significance of the effects on the 95 kDa form of α-ENaC is therefore not clear.

Brief (3 h) stimulation with dexamethasone

Although dexamethasone clearly activated SGK1, this response was not associated with any change to the activity of TORC2, and the situation seen in H441 cells is therefore similar to that documented for renal epithelia where the dexamethasone-induced activation of SGK1 seems to reflect *de novo* synthesis of SGK1 protein (Wang *et al.*, 2001; Gonzalez-Rodriguez *et al.*, 2007). However, as the nascent protein is catalytically inactive, the PI3K–TORC2 pathway is needed to phosphorylate SGK1-Ser^{422} and confer catalytic activity upon the newly synthesized protein (Kobayashi and Cohen, 1999; Park *et al.*, 1999; García-Martínez and Alessi, 2008; Lu *et al.*, 2010).

While undertaking these studies we noted glucocorticoid-deprived and dexamethasone-stimulated (24 h) cells often appeared to display very similar levels of NDRG1-Thr$^{346/356/366}$ phosphorylation, and this observation (see also Inglis *et al.*, 2009) prompted us to compare the effects of brief (3 h) and prolonged (24 h) dexamethasone stimulation upon the phosphorylation of these residues. These studies confirmed that brief exposure to dexamethasone did increase SGK1 activity without altering the activity of TORC2, but also showed that prolonged (24 h) stimulation had no apparent effect upon the activity of either kinase. Despite the sustained activation of ENaC, the present data therefore suggest that SGK1 activity returns to its basal level after ~24 h continuous exposure to dexamethasone. Moreover, experiments that explored the effects of brief (3 h) exposure to dexamethasone upon the surface abundance of ENaC subunits showed that the period of increased SGK1 activity was associated with an unambiguous increase in the surface abundance of α-, β- and γ-ENaC. Furthermore, this response was abolished by GSK650394 and it is therefore clear that SGK1 does provide a mechanism that allows hormonal control over the surface expression of ENaC subunits. However, despite this clear finding, electrophysiological studies showed that brief (3 h) exposure to dexamethasone did not induce Na^+ currents while studies of cells exposed to dexamethasone for ~24 h confirmed the sustained activation of endogenous ENaC as described earlier.

Significance of present findings

The conceptual model almost invariably used to explain the role of SGK1 in the control of epithelial Na^+ absorption (Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; De La Rosa *et al.*, 1999; 2003; Păunescu *et al.*, 2000; Debonneville *et al.*, 2001) predicts (i) that glucocorticoid-induced Na^+ currents will depend upon SGK1; and (ii) that the magnitude of this Na^+ current will correlate with the activity of SGK1 and with the abundance of ENaC subunits at the cell surface. Although data from the present study clearly verifies the first of these statements, they also show that the effects of dexamethasone upon SGK1 activity and the surface expression of ENaC subunits are transient and it is therefore clear that the sustained, SGK1-dependent activation of ENaC seen in glucocorticoid-stimulated H441 cells (present study Clunes *et al.*, 2004; Brown *et al.*, 2008; Althaus *et al.*, 2010) cannot be attributed to SGK1-dependent changes to the surface abundance of ENaC subunits. Studies of heterologously expressed ENaC have suggested that SGK1 can control membrane Na^+ current via a mechanism that does not appear to involve the recruitment of additional channel subunits to the membrane, and this response is dependent upon an SGK1-consensus motif in the C-terminal region of α-ENaC. It has been suggested that SGK1 may contribute to the control of ENaC activity by directly phosphorylating α-ENaC (Diakov and Korbmacher, 2004). However, this mechanism is unlikely to explain the sustained activation of ENaC reported here because our data indicate that glucocorticoid-induced SGK1 activity is not sustained.

The signalling pathways that allow glucocorticoids to evoke responses in pulmonary epithelia are complex (Barnes, 2011) and the present data show that the mechanisms that allow these hormones to evoke sustained activation of ENaC are not understood fully. Clarifying the mechanisms underlying this control over membrane Na^+ conductance may facilitate the developments of novel therapies for respiratory distress and pulmonary oedema.

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Conflict of interest

The authors report no conflict of interest.

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