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RESEARCH PAPER

Dysregulation of epithelial Na^+ absorption induced by inhibition of the kinases TORC1 and TORC2

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BACKGROUND AND PURPOSE
Although the serum and glucocorticoid-inducible protein kinase 1 (SGK1) appears to be involved in controlling epithelial Na^+ absorption, its role in this physiologically important ion transport process is undefined. As SGK1 activity is dependent upon target of rapamycin complex 2 (TORC2)-catalysed phosphorylation of SGK1-Ser^{422}, we have explored the effects of inhibiting TORC2 and/or TORC1 upon the hormonal control of Na^+ absorption.

EXPERIMENTAL APPROACH
Na^+ absorption was quantified electrometrically in mouse cortical collecting duct cells (mpkCCD) grown to confluence on permeable membranes. Kinase activities were assessed by monitoring endogenous protein phosphorylation, with or without TORC1/2 inhibitors (TORIN1 and PP242) and the TORC1 inhibitor: rapamycin.

KEY RESULTS
Inhibition of TORC1/2 (TORIN1, PP242) suppressed basal SGK1 activity, prevented insulin- and dexamethasone-induced SGK1 activation, and caused modest (10–20%) inhibition of basal Na^+ absorption and substantial (~80%) inhibition of insulin/dexamethasone-induced Na^+ transport. Inhibition of TORC1 did not impair SGK1 activation or insulin-induced Na^+ transport, but did inhibit (~80%) dexamethasone-induced Na^+ absorption. Arginine vasopressin stimulated Na^+ absorption via a TORC1/2-independent mechanism.

CONCLUSION AND IMPLICATIONS
Target of rapamycin complex 2, but not TORC1, is important to SGK1 activation. Signalling via phosphoinositide-3-kinase/TORC2/SGK1 can explain insulin-induced Na^+ absorption. TORC2, but not TORC1, is also involved in glucocorticoid-induced SGK1 activation but its role is permissive. Glucocorticoid-induced Na^+ transport displayed a requirement for TORC1 activity. Therefore, TORC1 and TORC2 contribute to the regulation of Na^+ absorption. Pharmacological manipulation of TORC1/2 signalling may provide novel therapies for Na^+-sensitive hypertension.

Abbreviations
APC, adenomatous polyposis; AVP, arginine vasopressin; CREB, cAMP response element binding protein; ENaC, epithelial sodium channel; GSK3, glycogen synthase kinase 3; I_{Eq}, equivalent short circuit current; IGF-1, insulin-like growth factor 1; mpkCCD, mouse cortical collecting duct cells; NDRG1, protein encoded by n-myc downstream regulated gene 1; Nedd-4/2, neural precursor cell expressed, developmentally down-regulated protein 4-2; P70-S6K, 70 kDa ribosomal S6 kinase; PDK1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide-3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKA, adenine-nucleotide-dependent protein kinase; PKB, protein kinase B (also known as Akt); R_t, transepithelial resistance; SGK1, serum and glucocorticoid-inducible protein kinase 1; TORC1 and 2, target of rapamycin complex 1 and 2; V_t, transepithelial voltage

Introduction

Phosphoinositide-3-kinase (PI3K) controls many aspects of cellular physiology by catalysing the formation of phospholipid second messenger compounds, most notably phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which regulate downstream protein kinases such as protein kinase B (PKB, also known as Akt) and serum and glucocorticoid-inducible kinase 1 (SGK1) (see Cohen, 2006). Early evidence that PI3K is involved in the control of epithelial Na^+ absorption came from the observation that PI3K inhibitors inhibit electrogenic Na^+ transport by reducing the number of functional, epithelial Na^+ channels (ENaC; nomenclature follows Alexander *et al.*, 2009) in the apical membrane (Paunescu *et al.*, 2000). This control over the apical ENaC expression appears to depend upon SGK1, a protein kinase that can prevent the internalization and degradation of ENaC subunits which normally seems to restrict apical Na^+ conductance (Kobayashi and Cohen, 1999; Park *et al.*, 1999; Debonneville *et al.*, 2001; Wang *et al.*, 2001; Snyder *et al.*, 2004b). Indeed, SGK1-dependent control over apical ENaC expression seems to allow PI3K-coupled agonists, such as insulin and insulin-like growth factor 1(IGF-1), to stimulate Na^+ transport (Blazer-Yost *et al.*, 1998; 2003; Kobayashi and Cohen, 1999; Park *et al.*, 1999; Debonneville *et al.*, 2001; Wang *et al.*, 2001; Snyder *et al.*, 2004b; Huang *et al.*, 2006; Gonzalez-Rodriguez *et al.*, 2007; Mansley and Wilson, 2010), and a body of evidence also implicates SGK1 in the control of Na^+ transport by mineralocorticoid and glucocorticoid hormones (Wang *et al.*, 1991; Itani *et al.*, 2002; Gonzalez-Rodriguez *et al.*, 2007). Moreover, SGK1 may also contribute to the control of Na^+ transport by cAMP/PKA (adenine-nucleotide-dependent protein kinase)-coupled hormones such as arginine vasopressin (AVP) (Gonzales-Robayana *et al.*, 2000; Perrotti *et al.*, 2001; Vasquez *et al.*, 2008). However, not all data are consistent with the idea that SGK1 plays such a central role in the control of Na^+ absorption as *sgk1* gene deletion causes only mild dysfunction of renal Na^+ handing (Wulff *et al.*, 2002) without preventing the regulation of colon Na^+ absorption (Rexhepaj *et al.*, 2006).

Whilst the role of PI3K/SGK1 in the hormonal control of Na^+ absorption is understood poorly (Lang *et al.*, 2006; Loffing *et al.*, 2006), it is clear that the PIP_3-dependent activation of this protein kinase depends upon the phosphorylation of SGK1-Ser^{422} by the target of rapamycin signalling complex 2 (TORC2) (García-Martínez and Alessi, 2008). Pharmacological inhibition of TORC2 therefore provides a novel and effective way of inactivating SGK1 (García-Martínez and Alessi, 2008). In order to clarify the role of SGK1 in the control of Na^+ absorption, we have, in the present study, explored the physiological consequences of exposing Na^+ absorbing, mouse cortical collecting duct cells (mpkCCD) to novel inhibitors of TORC2, TORIN1 (Thoreen *et al.*, 2009) and PP242 (Feldman *et al.*, 2009).

Methods

All methods have been described elsewhere (Mansley and Wilson, 2010) and so only brief details are given here.

Experiments were undertaken using confluent mpkCCD cells (Bens *et al.*, 1999) that had been deprived of hormones/growth factors for ~48 h. Previous work (Bens *et al.*, 1999) shows that these cells generate transepithelial current that is due, almost exclusively, to electrogenic Na^+ absorption and this parameter (equivalent short circuit current, I_{Eq}) was therefore monitored under open circuit conditions. All experiments were terminated by adding 10 μM amiloride to the apical bath, and this always caused >90% inhibition of I_{Eq} and, unless otherwise stated, also increased transepithelial resistance (R_t) one to twofold.

Changes in endogenous SGK1 activity were monitored by quantifying (by Western blot analysis and densitometry) the phosphorylation of residues ($Thr^{346/356/366}$) within the protein encoded by n-myc downstream regulated gene 1 (NDRG1) that are substrates for SGK1 but not for other closely related kinases (Murray *et al.*, 2005; Inglis *et al.*, 2009) whilst TORC1 and TORC2 activities were monitored by assaying the phosphorylation of the 70 kDa ribosomal S6 kinase (P70-S6K)-Thr^{389} (Proud, 2007) and protein kinase B (PKB)-Ser^{473} (Sarbassov *et al.*, 2005) respectively.

Data analysis

Experiments were undertaken using strictly paired protocols in which control and experimental cells were age-matched and at identical passage. Pooled data are shown as mean ± SEM and values of *n* refer to the number of times a protocol was repeated using cells at different passages. Statistical significances were tested using Student's *t*-test or analysis of variance (ANOVA), with Bonferroni *post hoc* test, as appropriate.

Materials

Amiloride, dexamethasone, AVP, insulin, culture reagents and general laboratory chemicals were from Sigma (Poole, Dorset, UK) whilst PP242 and TORIN1 were generous gifts from Prof D.R. Alessi (MRC Protein Phosphorylation Unit, University of

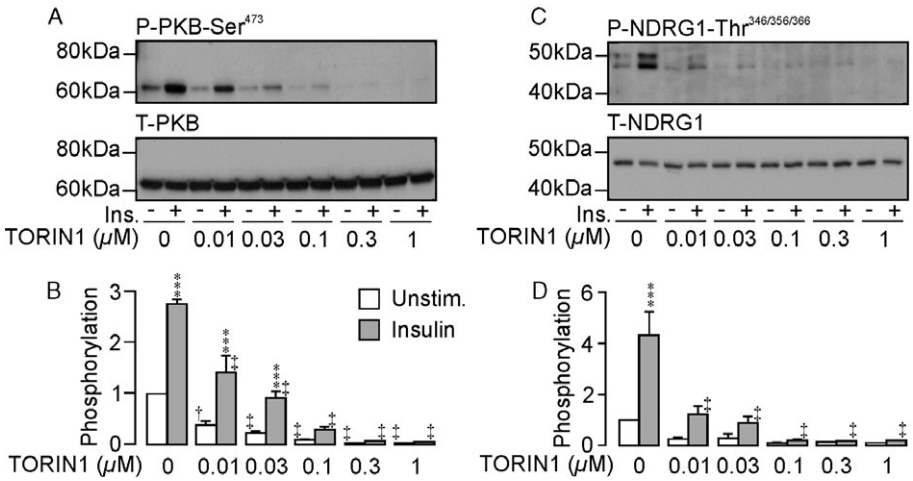

Figure 1

Effects of TORIN1 upon the phosphorylation of protein kinase B (PKB)-Ser^{473} and by n-myc downstream regulated gene 1-encoded protein 1 Thr$^{346/356/366}$ (NDRG1-Thr$^{346/356/366}$) in unstimulated (Unstim.) and insulin-stimulated (Ins.) cells. The blots in each figure illustrate the effects of TORIN 1 (0–1 μM, 30 min pre-incubation) upon the expression of (A) Ser^{473}-phosphorylated (upper panel) and total (lower panel) PKB, and (C) Thr$^{346/356/366}$-phosphorylated (upper panel) and total (lower panel) NDRG1, whilst the histograms include pooled data from six such experiments (mean ± SEM) which show the effects of insulin and/or TORIN1 upon the phosphorylation of PKB-Ser^{473} (B) and NDRG1-Thr $^{346/356/366}$ (D). ***$P < 0.01$; significant responses to insulin; Student's paired *t*-test. †$P < 0.05$; ‡$P < 0.02$; significant effects of TORIN1; ANOVA/Bonferroni *post hoc* test).

Dundee, Dundee, UK) and Prof D.M. Sabatini (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) respectively. Antibodies against Ser^{473}-phosphorylated and total PKB, Ser^{133}-phopsphorylated and total cAMP response element binding protein (CREB), and Thr^{389}-phosphorylated and total P70-S6K were from Cell Signalling (Hertfordshire, UK), whilst antibodies against Thr$^{346/356/366}$-phosphorylated and total forms of NDRG1 were generously provided by Prof D.R. Alessi.

Results

Insulin-induced phosphorylation of endogenous proteins

Hormone-deprived cells displayed basal phosphorylation of PKB-Ser^{473}, and insulin stimulation (20 nM, 30 min) increased the abundance of this phosphoprotein without altering the overall PKB expression level (Figure 1A,B), indicating (Sarbassov *et al.*, 2005) that this hormone normally activates TORC2. TORIN1 (0.03–1 μM, 30 min pre-incubation) caused a concentration-dependent decline in the abundance of Ser^{473}-phosphorylated PKB both in hormone-deprived and insulin-stimulated cells, and concentrations ≥0.1 μM were fully effective (Figure 1A). As TORIN1 did not alter the overall PKB expression level (Figure 1B), this response must reflect reduced abundance of the Ser^{473}-phosphorylated form of this protein. Although small responses to insulin persisted in the presence of 0.01 μM and 0.03 μM TORIN1, concentrations of TORIN1 ≥0.1 μM reduced the expression of Ser^{473}-phosphorylated PKB to an undetectable level in hormone-deprived and insulin-stimulated cells indicating complete inhibition of TORC2 (Figure 1A,B). Hormone-deprived cells also displayed clearly detectible levels of Thr$^{346/356/366}$-phosphorylated NDRG1, and insulin (20 nM, 30 min) also increased the abundance of this phosphoprotein without altering the overall expression level indicating activation of SGK1 (Figure 1C,D). TORIN1 reduced the abundance of Thr$^{346/356/3566}$-phosphorylated NDRG1 in hormone-deprived and insulin-stimulated cells without altering overall expression (Figure 1C,D) indicating inactivation of SGK1. This effect was complete at ≥0.1 μM.

Figure 2 shows the results of experiments that explored the effects of PP242 (1 μM, 30 min pre-incubation), another TORC2 inhibitor (Feldman *et al.*, 2009). These data confirm that insulin normally increases the abundance of both Ser^{473}-phosphorylated PKB and Thr$^{346/356/366}$-phosphorylated NDRG1, and also show that PP242 completely abolished the expression of these phosphoproteins in hormone-deprived and insulin-stimulated cells with no effect upon the expression of the corresponding full length proteins.

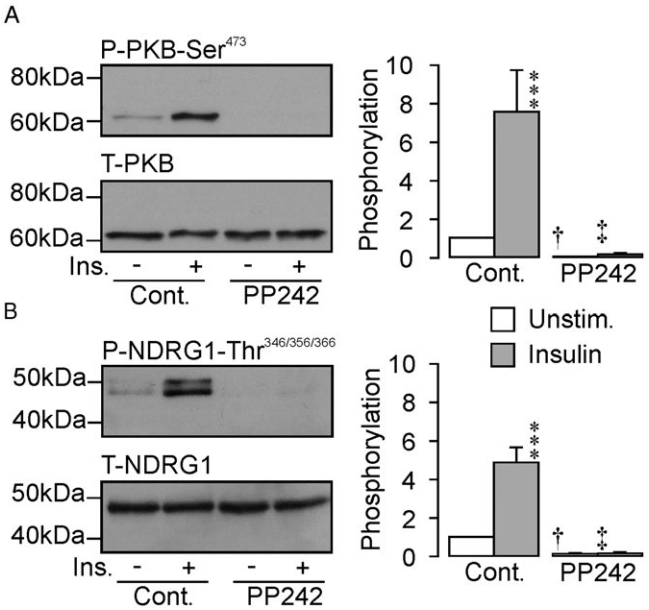

Figure 2

Effects of PP242 upon the phosphorylation of PKB-Ser^{473} and NDRG1-Thr$^{346/356/366}$. The blots in each figure illustrate the effects of PP242 (1 μM, 30 min pre-incubation) upon the abundance of (A) Ser^{473}-phosphorylated and total PKB and (B) Thr$^{346/356/366}$-phosphorylated and total NDRG1 in hormone-deprived and insulin-stimulated (20 nM, 30 min) cells, whilst the histograms each show the pooled data derived from six such experiments. ***$P < 0.01$, significant effects of insulin upon the phosphorylation of these endogenous proteins. †$P < 0.05$, ‡$P < 0.01$, significant effects of PP242; ANOVA/Bonferroni *post hoc* test). Cont., control; Ins., insulin-stimulated; NDRG1, protein encoded by n-myc downstream regulated gene 1, PKB, protein kinase B; Unstim., unstimulated.

Basal Na+ transport

Analysis of currents recorded from hormone-deprived cells showed that TORIN1 (0.1 μM) and PP242 (1 μM) caused slight (TORIN1: 10.8 ± 3.1%, $n = 32$; PP242: 22.3 ± 2.1%, $n = 33$) inhibition of electrogenic Na^+ absorption. These effects became apparent after 2–3 min and developed over the following 10–15 min. Rapamycin (0.1 μM, $n = 3$), an inhibitor of TORC1, had no effect upon the currents recorded under such conditions. These compounds had no discernible effect upon R_t indicating that epithelial integrity is maintained.

Insulin-stimulated Na+ transport

Figure 3A shows currents recorded from hormone-deprived and insulin-stimulated (20 nM, basolateral) control cells whilst Figure 3B shows data from TORIN1-treated (0.1 μM, 30 min) cells. To ensure that the small effects on the basal current described above did not confound analysis of these data, these results are shown as deviations from the value of I_{Eq} measured over the 5 min period prior to hormone application. The magnitude of this basal current normally showed a slight tendency to decline with time but insulin consistently evoked an augmentation of I_{Eq} that became apparent after ~5 min and reflected a hyperpolarization of the transepithelial voltage (V_t) that coincided with a small fall in R_t (not shown). The magnitude of this response was quantified by measuring the insulin-induced change in I_{Eq} and subtracting the spontaneously developing change measured in hormone-deprived cells. This analysis showed that insulin normally enhances the recorded current (Figure 3C) and, although a small response persisted in TORIN1-treated cells (Figure 5B, $P < 0.05$), the magnitude of this response was reduced by 73.3 ± 3.6%. Further experiments confirmed this effect of insulin (Figure 3D) and showed that PP242 (Figure 3E) caused 83.7 ± 6.7% inhibition of this response (Figure 3F). The magnitude of this effect did not differ from that quantified in TORIN1-treated cells.

Effects of aldosterone

Aldosterone could augment the amiloride-sensitive I_{Eq} by inducing a hyperpolarization of V_t that developed over 1–2 h and coincided with a small fall in R_t, and this hormone also increased the abundance of Thr$^{346/356/366}$-phosphorylated NDRG1 without altering the expression of the full length protein indicating activation of SGK1 (not shown). However, such responses occurred only in cells exposed to aldosterone concentrations 15- to 500-fold greater (0.5–5 μM) than those encountered *in vivo* and the cells were insensitive to physiologically relevant concentrations (1–30 nM) of this hormone.

Dexamethasone-induced protein phosphorylation

The control data in Figure 4 show that dexamethasone (0.2 μM, 2.5 h) had no effect upon the abundance of Ser^{473}-phosphorylated or total PKB (Figure 4A–C) indicating that this synthetic glucocorticoid did not activate TORC2. TORIN1 (0.1 μM, Figure 4A) and PP242 (1 μM, Figure 4B), on the other hand, reduced the expression of Ser^{473}-phosphorylated PKB to an undetectable level in both hormone-deprived and dexamethasone-stimulated cells, without altering the overall expression level. Rapamycin had no such effect (Figure 4C). Further experiments showed that dexamethasone consistently increased the abundance of the Thr$^{346/356/366}$-phosphorylated NDRG1 (Figure 4D–F) and, although this synthetic glucocorticoid had no effect upon the overall abundance of this protein, it did induce the appearance of a second, less mobile band in blots probed using the antibody against full length NDRG1 (Figure 4D–F).

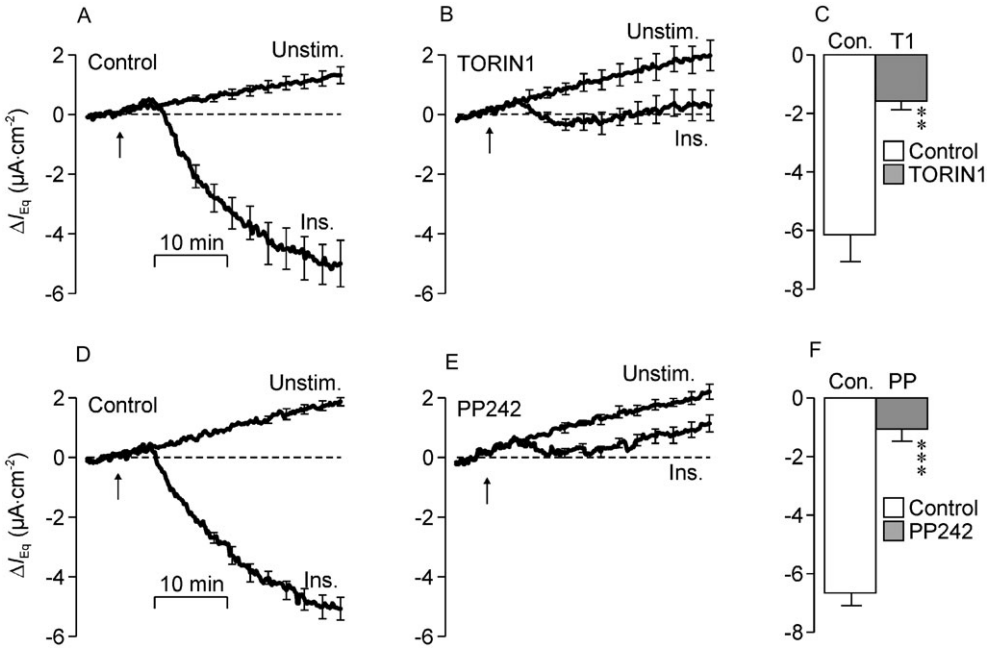

Figure 3

Effects of TORIN1 and PP242 upon the electrometric response to insulin. In this and in all subsequent such figures the values of equivalent short circuit current (I_{Eq}) measured during the initial 5 min of each recording period (i.e. the basal I_{Eq}) has been subtracted from all subsequently measured data points so that each presented trace shows the changes (mean ± SEM, $n = 5$) from this basal value (ΔI_{Eq}). (A) Values of I_{Eq} derived from unstimulated and insulin-stimulated (Ins.; 20 nM, basolateral, arrows) control cells. In this series of experiments the values of basal I_{Eq} quantified in the unstimulated (Unstim.) and insulin-stimulated cells were –14.4 ± 1.9 μA·cm^{-2} and –17.0 ± 2.0 μA·cm^{-2} respectively. (B) Data from age-matched cells pretreated (30 min) with 0.1 μM TORIN1 (basal I_{Eq} unstimulated cells: –13.6 ± 1.2 μA·cm^{-2}; basal I_{Eq} insulin-stimulated cells: –14.6 ± 1.4 μA·cm^{-2}). (C) Responses to insulin in control (Con.) and TORIN1-treated cells were quantified by measuring the change in I_{Eq} that developed during exposure to hormone and subtracting the spontaneous change measured in unstimulated cells. (D) Data showing the spontaneous and insulin-induced changes in I_{Eq} in control cells (basal I_{Eq} unstimulated cells: –17.5 ± 1.0 μA·cm^{-2}; basal I_{Eq} insulin-stimulated cells: –20.1 ± 1.6 μA·cm^{-2}). (E) Data from age-matched cells pretreated (30 min) with 1 μM PP242 (basal I_{Eq} unstimulated cells: –13.9 ± 1.1 μA·cm^{-2}; basal I_{Eq} insulin-stimulated cells: –15.7 ± 1.3 μA·cm^{-2}). (F) Responses to insulin measured in control and PP242-treated cells. $^{**}P < 0.01$; $^{***}P < 0.005$, significant responses to insulin; Student's paired *t*-test.

TORIN1 (Figure 4D) and PP242 (Figure 4E) virtually abolished the expression of Thr$^{346/356/366}$-phosphorylated NDRG1 in hormone-deprived and dexamethasone-stimulated cells without altering the overall expression level. Whilst a small response to dexamethasone persisted in the presence of TORIN1, this did not reach statistical significance (Figure 4D). Rapamycin (0.1 μM) did not alter the phosphorylation status of NDRG1-Thr$^{346/356/366}$ (Figure 4F). Further experiments ($n = 4$, not shown) showed that hormone-deprived cells normally displayed detectible levels of Thr^{389}-phosphorylated P70-S6K indicating basal activity of TORC1. Dexamethasone (0.2 μM, 2.5 h) had no effect upon the phosphorylation of this residue establishing that this synthetic steroid did not activate TORC1. Rapamycin (0.1 μM), on the other hand, abolished the expression of Thr^{389}-phosphorylated P70-S6K in hormone-deprived and dexamethasone-stimulated cells without altering the overall expression of this protein (not shown). Rapamycin therefore causes complete inactivation of TORC1 under the present conditions.

Dexamethasone-induced Na^+ transport

The control data in Figure 5 show that dexamethasone (0.2 μM) increased I_{Eq} although this response developed more slowly than the response to insulin (see above), as it only became apparent after ~40-min latency. Moreover, the present experiments were terminated after 2.5 h and the response had not reached a plateau value by this time (Figure 5A,D,G). Whilst there was some evidence of a dexamethasone-induced augmentation of I_{Eq} in TORIN1- (Figure 5B) and PP242- (Figure 5E) treated cells, these responses were smaller than control (Figure 5C,F) and analysis showed that these substances caused 87.4 ± 3.8% and 92.1 ± 3.9% inhibition respectively. Although rapamycin did not affect the dexamethasone-induced activation of SGK1 (Figure 4F), this substance suppressed the dexamethasone-induced Na^+ transport (Figure 5H)

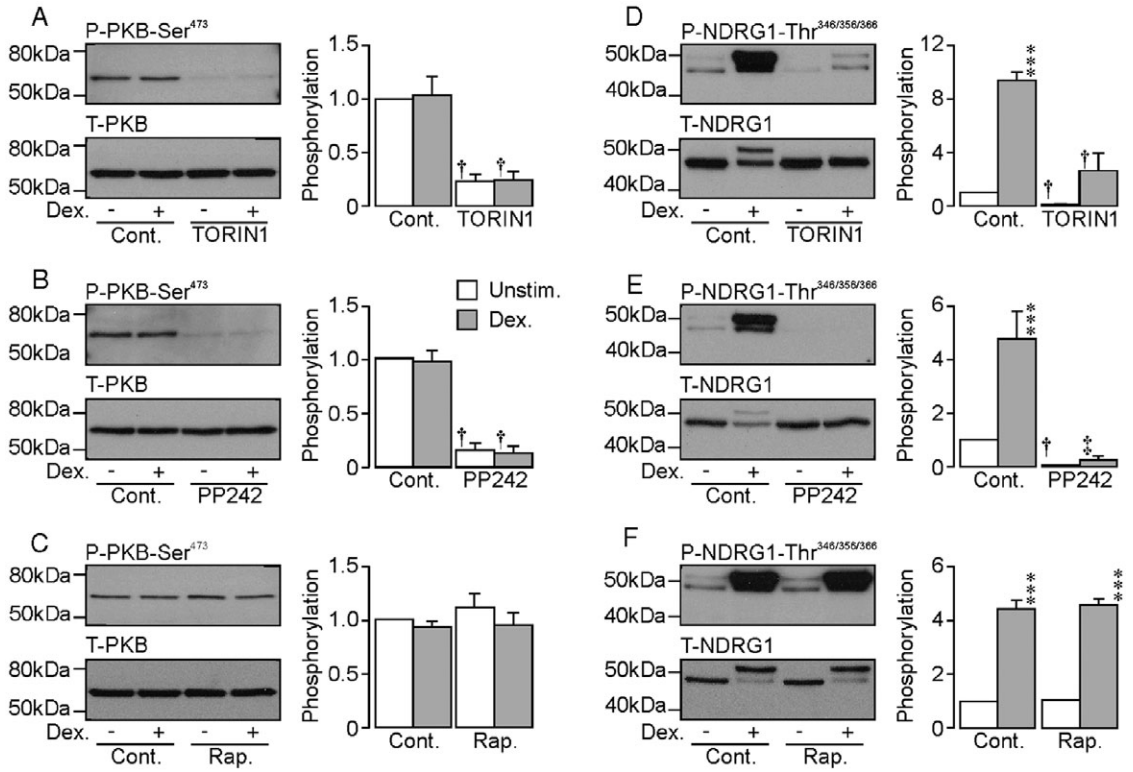

Figure 4

Effects of TORIN1 and PP242 upon the phosphorylation of PKB-Ser^{473} and NDRG1-Thr$^{346/356/366}$ in unstimulated and dexamethasone-stimulated cells. The blots in each figure illustrate the effects of 0.1 μM TORIN1 (A, D) 1 μM PP242 (B, E) and 0.1 μM rapamycin (C, F) upon the abundance of Ser^{473}-phosphorylated and total PKB (A, B, C) and Thr$^{346/356/366}$-phosphoryated and total NDRG1 (D, E, F) measured in both hormone-deprived and dexamethasone-stimulated (0.2 μM, 2.5 h) cells. The pooled data (mean ± SEM) presented in the histograms are each derived from a total of six (TORIN1, PP242) or four (rapamycin) such experiments. ***$P < 0.001$, significant responses to dexamethasone; Student's paired *t*-test. †$P < 0.05$; ‡$P < 0.001$, significant effects of the TORC1/2 inhibitors; ANOVA. Cont., control; Dex., dexamethasone-stimulated; NDRG1, protein encoded by n-myc downstream regulated gene 1, PKB, protein kinase B; Rap., rapamycin; Unstim., unstimulated.

as effectively (84.2 ± 3.4% inhibition) as TORIN1 (Figure 5B) or PP242 (Figure 5E).

Responses to AVP

Figure 6A shows continuous records of V_t derived from confluent cells. Standard pulses of transepithelial current (–10 μA·cm^{-2}, 20 s) were repeatedly injected throughout all such experiments so that R_t could be calculated, and analysis of these records showed that V_t was normally ~ –50 mV (Figure 6C) and, as R_t was ~2 kΩcm^2 (Figure 6D), I_{Eq} was normally ~ –25 μA·cm^{-2} (Figure 6F). Apical amiloride (10 μM) essentially abolished V_t and increased R_t, and only negligible current (–1.5 ± 0.1 μA·cm^{-2}) persisted in the presence of this ENaC blocker. Figure 6B shows that AVP induced a depolarization of V_t that became apparent immediately and developed over ~30 min (Figure 6C). However, this depolarization was accompanied by a substantial fall in R_t (Figure 6B,D), and analysis using Ohm's Law showed that AVP actually caused a substantial augmentation of I_{Eq} (Figure 6E). Subsequent application of amiloride essentially abolished V_t (Figure 6B) and reduced I_{Eq} to a negligible level (–2.2 ± 1.0 μA·cm^{-2}). Amiloride was added after 30 min exposure to AVP and the fact that this drug essentially abolishes the recorded current shows that the response to AVP measured at this time point must reflect a stimulation of electrogenic Na$^+$ transport. Amiloride did not, however, increase R_t in AVP-stimulated cells (Figure 6B) and the fall in resistance evoked by this peptide hormone cannot therefore be attributed to ENaC activation. The mechanism of the AVP-induced fall in R_t was not investigated further. AVP (10 nM, 30 min) also evoked a significant increase in the abundance of Ser^{133}-phosphorylated CREB that occurred with no change to overall expression of this protein (Figure 6F,G). As CREB-Ser^{133} is a substrate for PKA, this result shows that AVP activates the cAMP/PKA-dependent signalling pathway.

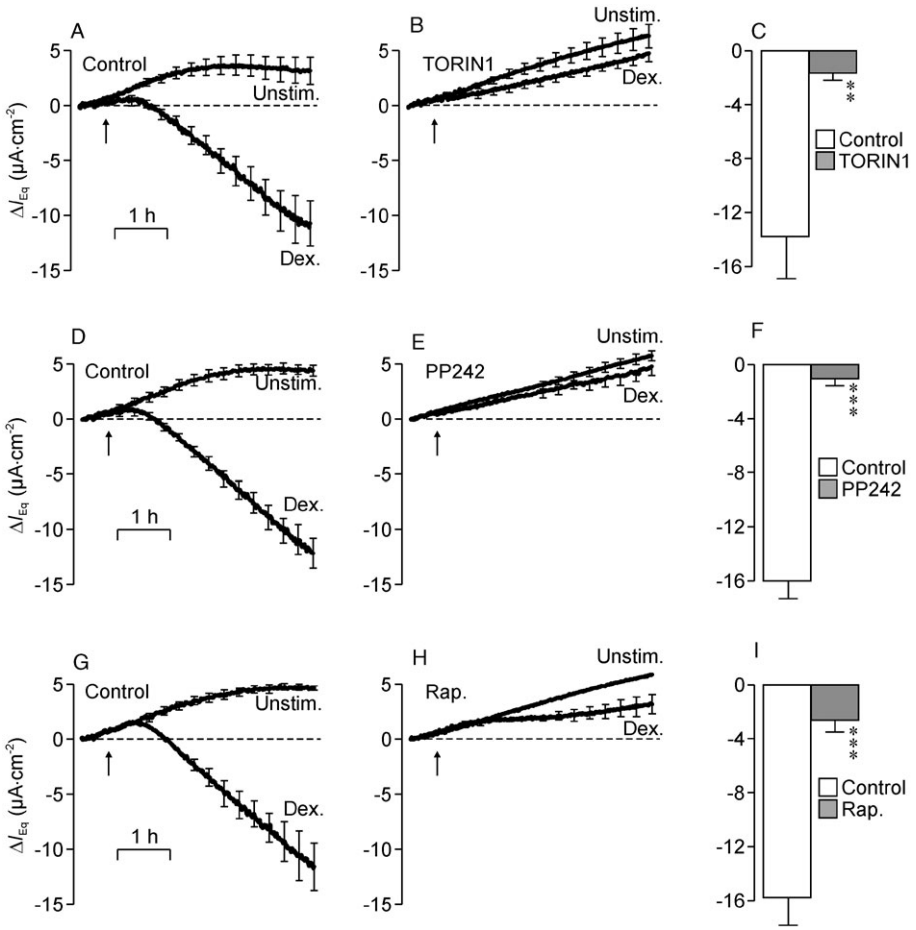

Figure 5

Effects of TORIN1, PP242 and rapamycin upon the dexamethasone-induced stimulation of Na^+ transport. (A) Changes in equivalent short circuit current (I_{Eq}) (n = 6) measured in unstimulated (Unstim.) and dexamethasone-stimulated (Dex.; 0.2 μM, arrows) cells maintained under control conditions (basal I_{Eq} unstimulated cells: –20.7 ± 1.9 μA·cm^{-2}; basal I_{Eq} dexamethasone-stimulated cells: –22.0 ± 2.8 μA·cm^{-2}). (B) Data from age-matched cells at identical passage pretreated (30 min) with 0.1 μM TORIN1 (basal I_{Eq} unstimulated cells: –15.9 ± 1.8 μA·cm^{-2}; basal I_{Eq} dexamethasone-stimulated cells: –18.9 ± 2.2 μA·cm^{-2}). (C) Responses to dexamethasone in control and TORIN1-treated cells were quantified by measuring the change in current that developed during exposure to this hormone and subtracting the spontaneously developing change in I_{Eq} measured in unstimulated cells. (D) Data (n = 6) from separate experiments showing spontaneous and dexamethasone-induced changes in I_{Eq} in control cells (basal I_{Eq} unstimulated cells: –20.8 ± 1.0 μA·cm^{-2}; basal I_{Eq} dexamethasone-stimulated cells: –20.8 ± 1.5 μA·cm^{-2}). (E) Data from age-matched pretreated (30 min) with 1 μM PP242 (basal I_{Eq} unstimulated cells: –14.8 ± 1.0 μA·cm^{-2}; basal I_{Eq} dexamethasone-stimulated cells: –14.7 ± 1.4 μA·cm^{-2}). (F) Responses to dexamethasone quantified in control and PP242-treated cells. (G) Spontaneous and dexamethasone-induced changes in I_{Eq} measured under control conditions (n = 3) (basal I_{Eq} unstimulated cells: –18.7 ± 0.2 μA·cm^{-2}; basal I_{Eq} dexamethasone-stimulated cells: –19.2 ± 1.7 μA·cm^{-2}). (H) Data from age-matched cells pretreated (30 min) with 0.1 μM rapamycin (Rap.; basal I_{Eq} unstimulated cells: –14.9 ± 1.0 μA·cm^{-2}; basal I_{Eq} dexamethasone-stimulated cells: –15.4 ± 1.9 μA·cm^{-2}). (I) Responses to dexamethasone in control and rapamycin-treated cells. **P < 0.02, ***P < 0.01, significant effects of the inhibitors; Student's paired t-test.

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

The control data in Figure 7 show AVP (10 nM, 30 min) slightly reduced (20–30%) the abundance of Thr$^{346/356/366}$-phosphorylated NDRG1 without altering overall expression level. The AVP-induced stimulation of Na^+ transport (Figure 6) thus appears to be associated with slight loss of SGK1 activity (Figure 7). Analysis of protein extracted from TORIN1- (Figure 7A,B) or PP242-treated (Figure 7C,D) cells confirmed that these substances abolished NDRG1-Thr$^{346/356/366}$ phosphorylation, and showed that AVP had no effect upon the phosphorylation status of these residues in TORIN1/PP242-treated cells (Figure 7A,B).

AVP-induced Na^+ transport

Figure 8 shows that the AVP induced augmentation of I_{Eq} developed more rapidly than the equivalent response to insulin and was preceded by a transient

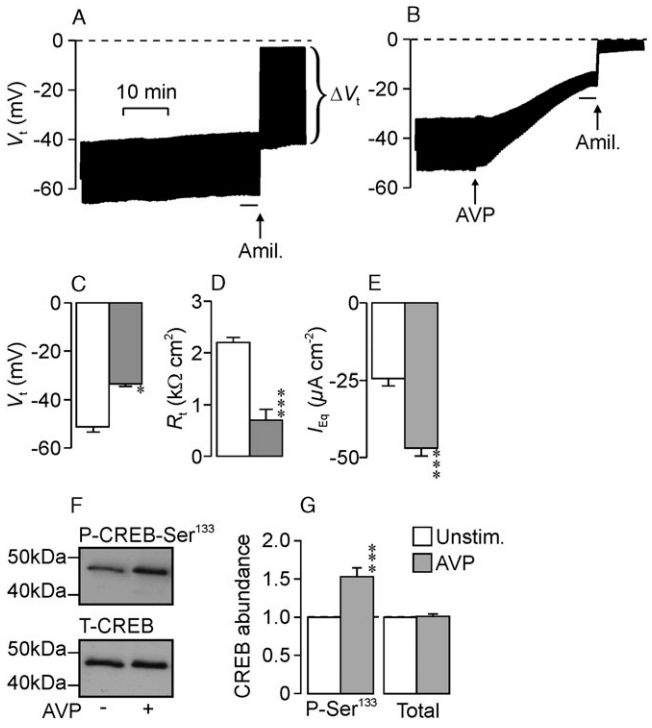

Figure 6

The response to arginine vasopressin (AVP). The upper panels show records of transepithelial voltage (V_t) derived from unstimulated (A) and AVP-treated (B, 10 nM, arrows) cells that were age-matched and at identical passage. Experiments were terminated by adding apical amiloride (Amil.; 10 μM, arrows). Throughout all such experiments pulses of transepithelial current (–10 μA·cm^{-2}, 20 s) were injected every minute so that the transepithelial resistance (R_t) could be determined ($R_t = \Delta V_t/\Delta I$) which allowed the equivalent short circuit current (I_{Eq}, i.e. the current required to hold V_t at 0 mV) to be calculated ($I_{Eq} = V_t/R_t$). The middle panels show pooled values (mean ± SEM) of V_t (C), R_t (D) and I_{Eq} (E) derived from a series ($n = 9$) of such experiments. These data are derived from analysis of data recorded immediately prior to the addition of amiloride. (F) Typical Western blots from experiments that explored the effects of AVP (10 nM, 30 min) upon the abundance of Ser^{133}-phosphorylated (upper panel) and total (lower panel) cAMP response element binding protein (CREB). (G) Pooled data showing the effects of AVP upon the abundance of Ser^{133}-phosphorylated (P-Ser^{133}) and total CREB ($n = 14$). *$P < 0.05$, ***$P < 0.001$, significant effects of AVP; Student's paired *t*-test.

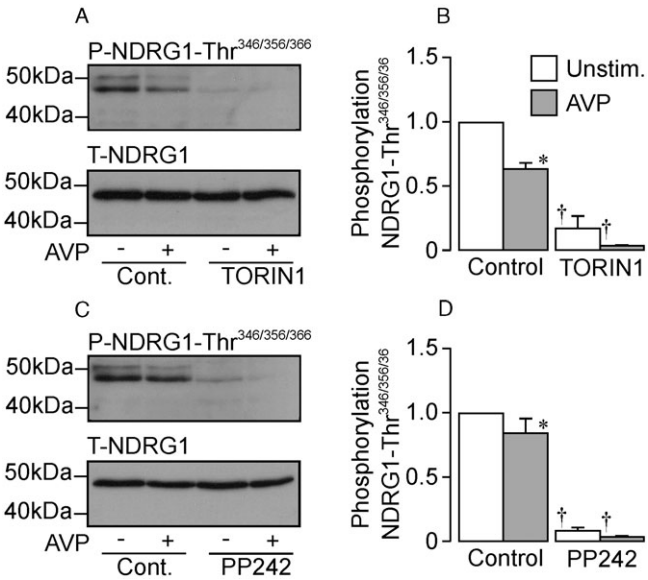

Figure 7

Effects of arginine vasopressin (AVP) upon the phosphorylation of Thr$^{346/356/366}$ in protein encoded by n-myc downstream regulated gene 1 (NDRG1) in control, TORIN1-treated and PP242-treated cells. (A) Typical Western blots showing the effects of AVP (10 nM, 30 min exposure) upon the abundance of the Thr$^{346/356/366}$-phosphorylated (upper panels) and total NDRG1 (lower panels) in control (Cont.) and TORIN1-pretreated (0.1 μM, 30 min) cells. (B) Pooled data showing the effects of AVP upon the abundance of Thr$^{346/356/366}$-phosphorylated NDRG1 in control and TORIN1-treated cells ($n = 6$). (C) Western blots showing the effects of AVP (10 nM, 30 min exposure) upon the abundance of the Thr$^{346/356/366}$-phosphorylated (upper panels) and total NDRG1 (lower panels) in control and PP242-pretreated (1 μM, 30 min) cells. (D) Pooled data showing the effects of AVP upon NDRG1-Thr$^{346/356/366}$ phosphorylation in control and PP242-treated cells ($n = 6$). *$P < 0.05$, significant effects of AVP; Student's paired *t*-test. †$P < 0.05$, significant effects of TORIN1 or PP242; one-way ANOVA, Bonferroni *post hoc* test). Unstim., unstimulated.

suppression of I_{Eq}. TORIN1 (Figure 8B) and PP242 (Figure 8F) had no effect upon this response (Figure 8C,G).

Discussion

Effectiveness of TORC2 inhibitors

The aim of initial experiments was to ensure that the TORC2 inhibitors were effective under the conditions used in the present study and, as TORC2 catalyses the PI3K-dependent phosphorylation of PKB-Ser^{473} (Sarbassov *et al.*, 2005), we therefore explored the effects of TORIN1 upon the phosphorylation of this residue. These studies showed that TORIN1 caused a concentration-dependent decline in the abundance of Ser^{473}-phosphorylated PKB in hormone-deprived and insulin-stimulated cells suggesting strongly that TORIN1 does suppress TORC2 activity in mpkCCD cells. However, there is substantial homology between the catalytic domains of TORC1, TORC2 and PI3K, which makes it difficult to design compounds that block these kinases selectively (Brunn *et al.*, 1993; Bain *et al.*, 2007). Moreover, inhibition of PI3K or TORC2 would both prevent the phosphorylation of PKB-Ser^{473} (Bayascas and Alessi, 2005; Sarbassov *et al.*, 2005), which raises the possibility that the present effect of TORIN1 may reflect direct inhibition of PI3K.

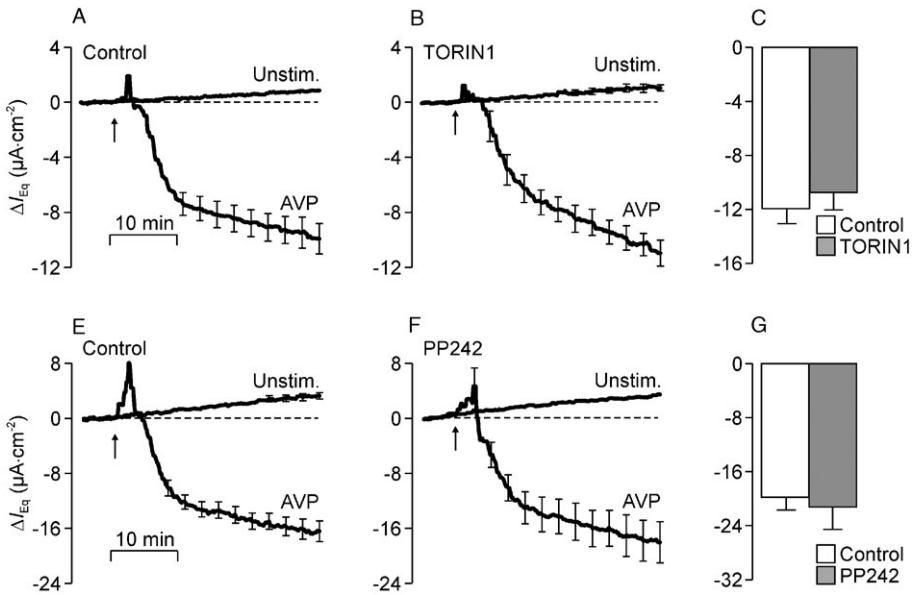

Figure 8

Effects of TORIN1 and PP242 upon the electrometric response to arginine vasopressin (AVP). (A) Changes in equivalent short circuit current (I_{Eq}) ($n = 4$) measured in studies of unstimulated (Unstim.) and AVP-stimulated (10 nM, basolateral, arrows) control cells (basal I_{Eq} unstimulated cells: -15.8 ± 0.6 μA·cm^{-2}; basal I_{Eq} AVP-stimulated cells: -13.1 ± 1.0 μA·cm^{-2}). (B) Data from age-matched cells that were pretreated (30 min) with 100 nM TORIN1 (basal I_{Eq} unstimulated cells: -12.8 ± 1.5 μA·cm^{-2}; basal I_{Eq} AVP-stimulated cells: -13.3 ± 2.0 μA·cm^{-2}). (C) Responses to AVP in control and TORIN1-treated cells were quantified by measuring the change in I_{Eq} that developed during exposure to AVP and subtracting the spontaneous change measured in unstimulated cells. (E) Data ($n = 5$) from separate control experiments showing the spontaneous and AVP-induced changes in I_{Eq} (basal I_{Eq} unstimulated cells: -27.5 ± 2.8 μA·cm^{-2}; basal I_{Eq} AVP-stimulated cells: -30.3 ± 1.4 μA·cm^{-2}). (F) Data from age-matched cells at identical passage that had been pretreated (30 min) with 1 μM PP242 (basal I_{Eq} unstimulated cells: -20.8 ± 1.8 μA·cm^{-2}; basal I_{Eq} AVP-stimulated cells: -22.4 ± 1.6 μA·cm^{-2}). (G) Responses to AVP quantified in control and PP242-treated cells.

Thoreen *et al*. (2009) addressed this issue by monitoring the phosphorylation of an alternative residue within PKB (Thr^{308}) that is catalysed in a PIP_3-dependent manner via a mechanism that does not involve TORC2 (Biondi *et al*., 2001; Bayascas and Alessi, 2005). However, as the TORC2-catalysed phosphorylation of PKB-Ser^{473} can allosterically enhance the phosphorylation of PKB-Thr^{308} (Biondi *et al*., 2001; Sarbassov *et al*., 2005), these studies were undertaken using cells lacking a critical component of the TORC2 complex to ensure that the phosphorylation status of PKB-Thr^{308} would provide a readout of PI3K activity (Thoreen *et al*., 2009). Whilst these studies showed that TORIN1 could inhibit PI3K, this was seen only in cells exposed to >1 μM TORIN1 whilst the inhibition of TORC2 was complete at <0.1 μM (Thoreen *et al*., 2009). The fact that the present effect on the phosphorylation status of PKB-Ser^{473} was complete at 0.1 μM TORIN1 therefore shows that this cannot be due to inhibition of PI3K. Moreover, although TORIN1 inhibits TORC1 and TORC2 (Thoreen *et al*., 2009), it is clear that rapamycin, an inhibitor of TORC1 (Bain *et al*., 2007), does not alter the phosphorylation status of PKB-Ser^{473} (present study, Sarbassov *et al*., 2005; Mansley and Wilson, 2010). Moreover, experiments that monitored the phosphorylation status of P70-S6K-Thr^{389} clearly showed that rapamycin did cause complete inhibition of TORC1 under the present conditions (Mansley and Wilson, 2010), whilst detailed *in vitro* studies have shown that, even when used at 1 μM, rapamycin does not suppress the activity of any member of a panel of 70 different protein kinases. It is therefore clear that rapamycin is an extremely selective inhibitor of the TORC1 signalling complex (Bain *et al*., 2007). The present data, in common with earlier findings (Thoreen *et al*., 2009) therefore show that TORC2 is fully inactivated by 0.1 μM TORIN1, and the fact that TORIN1 also blocked the phosphorylation of an endogenous SGK1 substrate (NDRG1-Thr$^{346/356/366}$) supports the view that TORC2 is critical to the PI3K-dependent activation of SGK1 (García-Martínez and Alessi, 2008). Further evidence of this comes from the fact that PP242, a structurally unrelated compound that also inhibits TORC1/2 (Feldman *et al*., 2009), reproduced these effects of TORIN1. Exposing cells to TORIN1/PP242 therefore causes essentially complete loss of SGK1 activity, and subsequent experiments therefore

explored the effects of these compounds upon the hormonal control of electrogenic Na^+ absorption.

Hormone-deprived cells

Hormone-deprived mpkCCD cells generate I_{Eq} via an apparently spontaneous mechanism dependent upon ENaC (Bens *et al.*, 1999; Mansley and Wilson, 2010) and, whilst the TORC2 inhibitors used in the present study caused full inactivation of SGK1 (see above), these substances caused only 10–20% inhibition of basal Na^+ transport. As rapamycin was ineffective, these small effects must reflect inhibition of TORC2 and, because highly selective inhibitors of PI3K and SGK1 also have little effect upon basal Na^+ absorption (Mansley and Wilson, 2010), it now appears that signalling via PI3K – TORC2 – SGK1 makes only a very small contribution to this basal Na^+ transport process in this cell type. This accords well with the observation that deletion of the *sgk1* gene does not alter renal Na^+ handling in Na^+ replete animals (Wulff *et al.*, 2002).

The response to insulin

Insulin induced a clear stimulation of electrogenic Na^+ transport that was accompanied by unambiguous activation of TORC2 and SGK1 (see also Mansley and Wilson, 2010) and these findings accord well with the view that insulin stimulates Na^+ absorption within the distal nephron in addition to its well characterized role in carbohydrate metabolism (see e.g. Atchley *et al.*, 1936; Miller and Bogdonoff, 1954; Defronzo *et al.*, 1975; Tiwari *et al.*, 2007; Loffing and Korbmacher, 2009). TORIN1 and PP242 completely abolished the insulin-induced activation of TORC2 and SGK1 and also reduced (~80% inhibition) the stimulation of Na^+ transport. As these responses to insulin were unaffected by rapamycin (Mansley and Wilson, 2010), these data show that this hormone stimulates Na^+ absorption by activating the PI3K/TORC2/SGK1 pathway. This accords well with earlier data which show that insulin stimulates the trafficking of ENaC to the apical membrane via a mechanism dependent upon PI3K/SGK1 (see e.g. Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; Wang *et al.*, 2001; Arteaga and Canessa, 2006), and with the observation that *sgk1* gene deletion blocks insulin-induced Na^+ retention *in vivo* (Huang *et al.*, 2006). However, whilst TORIN1 and PP242 effectively abolished SGK1 activity, small responses to insulin did persist in TORIN1/PP242-treated cells. Whilst the physiological basis of these residual responses was not investigated, TORC1/2 inhibition would not prevent PI3K-catalysed formation of PIP_3 (Feldman *et al.*, 2009; Thoreen *et al.*, 2009) and such phospholipid second messengers can directly control ENaC activity (see e.g. Yue *et al.*, 2002; Markadieu *et al.*, 2004). It is therefore possible that PIP_3 may mediate the residual responses to insulin in TORIN1/PP242-treated cells.

Effects of aldosterone

In our hands, mpkCCD cells were insensitive to physiologically relevant concentrations of aldosterone and this contrasts with the situation documented in the original description of this cell line which documents responses to 0.1–50 nM aldosterone (Bens *et al.*, 1999). However, even in this study, high aldosterone concentrations (0.5 μM) were needed to achieve a maximal response and these authors concluded that the effects of this hormone were largely mediated via glucocorticoid receptors rather than via mineralocorticoid receptors (Bens *et al.*, 1999) and such high concentrations of aldosterone have been used in subsequent studies of these cells (see e.g. Lu *et al.*, 2010). The fact that we observe clear responses to 0.2 μM dexamethasone, a selective glucocorticoid receptor agonist, confirms that functional glucocorticoid receptors are present, and the reason why we did not see responses to physiological concentrations of aldosterone is unknown. However, aldosterone regulates Na^+ transport in the distal nephron by binding to mineralocorticoid receptors, and the simplest explanation of our data is that the cellular expression of such receptors may not be maintained *in vitro*. Although this suggestion raises the possibility that such permanent cell lines may display modified responses to the other Na^+ retaining hormones, it is clear that functional glucocorticoid receptors are present in the aldosterone-sensitive epithelia of the distal nephron (Loffing and Korbmacher, 2009). Indeed, glucocorticoid-receptor mediated Na^+ retention may contribute to the development of hypertension in pathophysiological conditions, such as obesity and chronic stress, characterized by over activity of the hypothalamic – pituitary – adrenal axis (Bailey *et al.*, 2009). Our subsequent experiments therefore characterize the effects of TORIN1/PP242 upon the response to this dexamethasone in order to define the role of TORC2 in such glucocorticoid receptor mediated Na^+ transport.

The response to dexamethasone

Dexamethasone caused robust activation of SGK1 and this response was anticipated because SGK1 was originally identified as a glucocorticoid-inducible protein kinase (Lang *et al.*, 2006; Loffing *et al.*, 2006). This response was essentially abolished by TORIN1/PP242 but unaffected by rapamycin, and this activation of SGK1, in common with the response to insulin, therefore depends upon TORC2

and not TORC1. However, whilst insulin consistently caused a clear increase in TORC2 activity, the glucocorticoid-induced activation of SGK1 did not coincide with any discernible change to the activity of this kinase. Dexamethasone and insulin therefore activate SGK1 via different molecular mechanisms and it is therefore interesting that dexamethasone does seem to increase the expression of SGK1 protein far more effectively than insulin (Wang *et al.*, 2001; Gonzalez-Rodriguez *et al.*, 2007). This would be expected as the promoter region of SGK1 gene contains a glucocorticoid response element that would allow dexamethasone to regulate transcription (Itani *et al.*, 2002). However, even if the response to dexamethasone was entirely dependent upon the *de novo* synthesis SGK1, TORC2 would still be needed because it is this kinase which confers catalytic activity upon the newly synthesized protein (Kobayashi and Cohen, 1999; Park *et al.*, 1999; García-Martínez and Alessi, 2008). Whilst TORC2 is essential to the glucocorticoid-induced activation of SGK1, our data therefore suggest that the role of this signalling complex is permissive and it is therefore interesting that work recently published by Lu *et al.* (2010) shows that PP242 can inhibit the current generated by mpkCCD cells that have been exposed to a combination of aldosterone and insulin. As rapamycin did not alter this current, these authors also concluded that TORC2 is critical to the hormonal control of ENaC function in these cells. Moreover, these authors (Lu *et al.*, 2010) showed that this hormone-stimulated current is also inhibited by suppressing the expression of a critical component of the TORC2 signalling complex, and present evidence confirms (García-Martínez and Alessi, 2008) that it is TORC2, and not TORC1, that catalyse the phosphorylation of SGK1-Ser^{422}. All data in this report are derived from cells exposed to aldosterone at a concentration (1 μM) much higher than that encountered *in vivo* and the responses described in this study will therefore be mediated, at least in part, via glucocorticoid receptors (see e.g. Bens *et al.*, 1999).

Although it did not alter the amount of NDRG1 in the cells, dexamethasone induced the appearance of a second, less mobile band on blots probed with the antibody against the full length protein. Two such bands were also identified in protein extracted from HeLa cells (Murray *et al.*, 2005), and it appears that the phosphorylation of NDRG1-Thr$^{346/356/366}$ converts this protein into a substrate for glycogen synthase kinase 3 (GSK3) which can then phosphorylate NDRG1 at Ser$^{342/352/362}$. The second, less mobile band seems to correspond to the GSK3-phosphorylation form of NDRG1 (Murray *et al.*, 2005), and the fact that dexamethasone, but not insulin, induced the formation of this band raises the possibility that glucocorticoids might activate GSK3 as well as SGK1.

Dexamethasone caused a clear and consistent stimulation of Na^+ absorption and, although this response developed more slowly than the response to insulin, the responses to both hormones were antagonized by TORIN1/PP242. However, whilst the response to insulin was unaffected by rapamycin (Mansley and Wilson, 2010), this TORC1 inhibitor did suppress the dexamethasone-induced stimulation of Na^+ transport, despite the fact that it did not prevent the accompanying activation of SGK1. This surprising result therefore identifies a further difference between the mechanisms that allow insulin and dexamethasone to regulate Na^+ transport and, despite the evidence implicating SGK1 in the control of Na^+ transport, the present data show that the glucocorticoid-induced activation of SGK1 does not provide a stimulus sufficient to drive increased Na^+ absorption. Although the hormone-sensitive currents described by Lu *et al.* (2010) were insensitive to rapamycin, the cells used in this study were all exposed to a high concentration aldosterone in combination with insulin, and this experimental design implies that a specific effect of rapamycin upon the responses to steroid hormones may not have been detected.

The mechanisms that allow TORC1 to contribute to the glucocorticoid-dependent control of Na^+ transport is unknown although it may be relevant that steroid hormones activate other signalling pathways involved in the regulation of ENaC activity. Such hormones thus evoke expression of glucocorticoid-inducible leucine zipper proteins (GILZ1-3) that appear to stimulate Na^+ transport by suppressing the activity of extracellular-signal regulated kinases (ERK1/2) (Soundararajan *et al.*, 2005). Moreover, it has recently been suggested that GILZ1 and SGK1 act synergistically to increase the abundance of ENaC in the plasma membrane (Soundararajan *et al.*, 2009). Since our data suggest that inhibition of TORC1 suppresses glucocorticoid-induced Na^+ transport without preventing the activation of SGK1, it is possible that TORC1 may play a role in the induction of GILZ1. Otulakowski *et al.* (2007), on the other hand, report that α-ENaC protein expression is dependent upon a signalling mechanism downstream to TORC1, and their studies of foetal distal lung epithelial cells, in common with the present data, show that electrogenic Na^+ transport can be inhibited by rapamycin under certain conditions. Further evidence of a role for TORC1 comes from studies with mice with a mutation in the adenomatous polyposis (APC) gene. This mutation induces a complex phenotype that

includes hyper-absorption of Na^+ in the colon (Koehl *et al.*, 2010) and it is therefore relevant that APC gene mutation causes activation of TORC1 and that the apparent hyper-activation of ENaC is corrected by rapamycin (Koehl *et al.*, 2010). Although the mechanism is obscure, at least two other lines of evidence implicate TORC1 in the control of ENaC function (Otulakowski *et al.*, 2007; Koehl *et al.*, 2010).

The response to AVP

Arginine vasopressin evoked changes in V_t and R_t that were very different to those induced by insulin (Mansley and Wilson, 2010) or dexamethasone as this hormone caused a depolarization of V_t that was associated with a marked fall in R_t. However, further analysis clearly showed that AVP did increase amiloride-sensitive I_{Eq} and this accords with the body of work indicating that this peptide hormone exerts a Na^+ retaining action in addition to its better-characterized anti-diuretic effect. Perhaps the best evidence of this comes from recent studies of micro-dissected mouse cortical collecting ducts which revealed acute (2–3 min) activation of ENaC in response to AVP and showed that this response was dependent upon the cAMP/PKA-dependent signalling pathway (Bugaj *et al.*, 2009). Studies of several renal epithelial cell lines have similarly shown that the Na^+ retaining effect of AVP is mediated via cAMP/PKA (see Loffing and Korbmacher, 2009) and our observation that AVP evokes CREB-Ser^{133} phosphorylation provides further evidence of this. However, despite these clear findings, studies of A6 cells suggest that PI3K/SGK1 may play a critically important role in this response (Edinger *et al.*, 1999), and cAMP-coupled hormones do seem to stimulate SGK1 expression/phosphorylation in ovarian granulosa cells (Gonzales-Robayana *et al.*, 2000) whilst PKA appears able to activate recombinant SGK1 (Perrotti *et al.*, 2001). It has therefore been suggested that cAMP/PKA-coupled agonists might control Na^+ transport by activating SGK1 (Perrotti *et al.*, 2001; Thomas *et al.*, 2004; Vasquez *et al.*, 2008).

The present data, however, show that AVP-induced Na^+ transport is not associated with increased phosphorylation of NDRG1-$Thr^{346/356/366}$. Indeed, AVP actually reduced the abundance of this phosphoprotein suggesting slight inhibition of SGK1. Moreover, although TORIN1 and PP242 clearly inactivated SGK1, these substances did not alter AVP-induced Na^+ transport and our data therefore indicate that cAMP-coupled agonists stimulate Na^+ absorption via a mechanisms completely independent of SGK1 (Inglis *et al.*, 2009). It is therefore interesting that Snyder *et al.* (2004a) have shown that LY294002, an inhibitor of PI3K, does not prevent the cAMP-induced activation of ENaC expressed in Fisher rat thyroid cells. Indeed, these authors suggested that PKA stimulates Na^+ transport by phosphorylating and inactivating neural precursor cell expressed, developmentally down-regulated protein 4-2 (Nedd-4/2), a ubiquitin ligase that can target the ENaC channel complex for internalization/degradation. Since Nedd-4/2 is also phosphorylated by SGK1 (Debonneville *et al.*, 2001; Snyder *et al.*, 2002), this protein may represent an important point of convergence between the PI3K and the PKA-dependent pathways (Snyder *et al.*, 2004a).

Significance of present findings

The regulated re-absorption of Na^+ within the distal nephron determines the amount of Na^+ lost in urine and is therefore critical to whole body Na^+ and water balance and to the control of blood pressure. Inappropriate stimulation of this Na^+ transport process underlies several forms of hypertension (Loffing and Korbmacher, 2009) and may complicate the clinical management of patients with type 2 diabetes who receive insulin-sensitizing drugs (Buckingham and Hanna, 2007). Our data confirm that TORC2 is of central importance to SGK1 activity (García-Martínez and Alessi, 2008) and indicate that insulin stimulates Na^+ transport by activating the PI3K – TORC2 – SGK1 pathway. Whilst this pathway is also essential to the glucocorticoid-induced activation of SGK1, its role in this response appears to be entirely permissive, whilst AVP-induced Na^+ transport seems to involve a separate mechanism. Moreover, whilst TORC1 activity is not needed for the insulin-dependent control of Na^+ transport, TORC1 does seem to be essential for glucocorticoid-induced Na^+ retention. Future studies must therefore clarify the way in which TORC1 contributes to the control of Na^+ transport by glucocorticoids and use alternative experimental systems that are sensitive to physiologically relevant concentrations of aldosterone, to define the role/s of TORC1/2 in the response to this critically important hormone. The present data do, however, raise the possibility of treating Na^+-sensitive hypertension by pharmacological manipulation of TORC1/2.

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Conflict of interest

The authors report no conflicts of interest.

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