

RESEARCH PAPER

Effects of nominally selective inhibitors of the kinases PI3K, SGK1 and PKB on the insulin-dependent control of epithelial Na⁺ absorption

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BACKGROUND AND PURPOSE

Insulin-induced Na⁺ retention in the distal nephron may contribute to the development of oedema/hypertension in patients with type 2 diabetes. This response to insulin is usually attributed to phosphatidylinositol-3-kinase (PI3K)/serum and glucocorticoid-inducible kinase 1 (SGK1) but a role for protein kinase B (PKB) has been proposed. The present study therefore aimed to clarify the way in which insulin can evoke Na⁺ retention.

EXPERIMENTAL APPROACH

We examined the effects of nominally selective inhibitors of PI3K (wortmannin, PI103, GDC-0941), SGK1 (GSK650394A) and PKB (Akti-1/2) on Na⁺ transport in hormone-deprived and insulin-stimulated cortical collecting duct (mpkCCD) cells, while PI3K, SGK1 and PKB activities were assayed by monitoring the phosphorylation of endogenous proteins.

KEY RESULTS

Wortmannin substantially inhibited basal Na⁺ transport whereas PI103 and GDC-0941 had only very small effects. However, these PI3K inhibitors all abolished insulin-induced Na⁺ absorption and inactivated PI3K, SGK1 and PKB fully. GSK650394A and Akti-1/2 also inhibited insulin-evoked Na⁺ absorption and while GSK650394A inhibited SGK1 without affecting PKB, Akti-1/2 inactivated both kinases.

CONCLUSION AND IMPLICATIONS

While studies undertaken using PI103 and GDC-0941 show that hormone-deprived cells can absorb Na⁺ independently of PI3K, PI3K seems to be essential for insulin induced Na⁺ transport. Akti-1/2 does not act as a selective inhibitor of PKB and data obtained using this compound must therefore be treated with caution. GSK650394A, on the other hand, selectively inhibits SGK1 and the finding that GSK650394A suppressed insulin-induced Na⁺ absorption suggests that this response is dependent upon signalling via PI3K/SGK1.

Abbreviations

Akti-1/2, inhibitor of Akt (also known as PKB) 1/2; ANOVA, analysis of variance; CK2, casein kinase 2; CCD, cortical collecting duct; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle's media; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; EIPA, 5-(N-ethyl-N-isopropyl) amiloride; ENaC, epithelial sodium channel; FBS, foetal bovine serum; G_{Na} , membrane Na⁺ conductance; GILZ1-3, glucocorticoid-inducible leucine zipper proteins 1-3; GLUT4, glucose transporter 4; GSK3, glycogen synthase kinase 3; HIPK, homeodomain-interacting protein kinase; I_{Eq} , equivalent short circuit current; I_{sc} , short circuit current; IC_{50} , concentration needed for 50% inhibition; Nedd-4/2, neural precursor cell expressed, developmentally down-regulated protein 4-2; NDRG1 and 2, protein encoded by n-myc downstream regulated gene 1

and gene 2; PDK1, phosphoinositide-dependent protein kinase 1; PH domain, plekstrin homology domain; PI3K, phosphatidylinositol-3-kinase; PIMK, provirus integration site for Moloney murine leukaemia virus kinase; PIP₂, phosphatidylinositol 3,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B (also known as Akt); PLK1, polo-like kinase 1; PRAS40, 40 kDa proline-rich substrate of Akt (PKB); P70-S6K, 70 kDa ribosomal S6 kinase; R_t, transepithelial resistance; SDS, sodium dodecyl sulphate; SGK1, serum and glucocorticoid-inducible kinase 1; SmMLCK, smooth-muscle myosin light-chain kinase; TORC1 and 2, target of rapamycin complex 1 and 2; V_t, transepithelial voltage

Introduction

The regulated re-absorption of Na⁺ within the aldosterone-sensitive epithelia of the distal nephron (ASDN; late distal convoluted tubule and the cortical collecting duct) is a physiologically important process that determines the amount of Na⁺ lost in urine, and this absorptive mechanism is therefore of central importance to Na⁺ homeostasis, whole body fluid balance and the control of blood pressure (reviewed by Loffing and Korbmaier, 2009). Na⁺ absorption within the ASDN is dependent upon epithelial Na⁺ channels (ENaC), transport proteins composed of 3 subunits (α -, β - and γ -ENaC) that form the highly selective Na⁺ channels, which allow apical Na⁺ entry in absorptive epithelia (Canessa *et al.*, 1993; 1994). The activity and/or surface expression of these channels is controlled by the hormones involved in the control of Na⁺ and water balance (aldosterone and vasopressin) but, in addition to its role in carbohydrate metabolism, insulin is also known to stimulate Na⁺ absorption within the ASDN (Atchley *et al.*, 1936; DeFronzo *et al.*, 1975; 1976; Blazer-Yost *et al.*, 1998; Tiwari *et al.*, 2007; Loffing and Korbmaier, 2009). While this effect may normally have little physiological significance (Loffing and Korbmaier, 2009), it can become relevant in patients with type 2 diabetes, as this condition is almost invariably treated using drugs that exert a generalized insulin-sensitizing action. At least some such drugs can cause tissue oedema, high blood pressure and increased risk of congestive heart failure, particularly if co-administered with insulin itself. While the mechanism underlying this oedema is not well understood (see review Buckingham and Hanna, 2007), it does seem to involve inappropriate stimulation of renal Na⁺ retention (Hong *et al.*, 2003; Guan *et al.*, 2005). Ideally, type 2 diabetes should therefore be treated using drugs that promote glucose uptake without altering renal Na⁺ handling, but this goal will only become achievable once the signalling pathways that allow insulin to control Na⁺ transport are fully understood. The present study therefore explores the mechanisms that allow insulin to stimulate Na⁺ transport in a cell line (mpkCCD) derived from

the mouse cortical collecting duct (Bens *et al.*, 1999).

Methods

Cell culture

Standard techniques were used to maintain the mpkCCD cell line, which is derived from the mouse cortical collecting duct (Bens *et al.*, 1999), in serial culture. The medium used was phenol red-free DMEM/Ham's F12 supplemented with glutamine (2 mM), foetal bovine serum (FBS, 2%, v/v), penicillin/streptomycin mixture (1%; Sigma, Poole, Dorset, UK), sodium selenate (60 nM), transferrin (5 μ g·mL⁻¹); dexamethasone (50 nM), triiodothyronine (1 nM), epidermal growth factor (10 ng·mL⁻¹) and insulin (5 μ g·mL⁻¹). For experiments, cells were removed from culture flasks using trypsin/EDTA and plated onto Snapwell membranes (5 \times 10⁵ cells·cm⁻², electrometric studies) or Transwell membranes (2.5 \times 10⁶ cells·cm⁻², Western analysis). Cells were then cultured in complete medium that was replaced every 48 h, and after 6 days, this standard medium was replaced with hormone-free medium (phenol red-free DMEM/Ham's F12 medium containing antibiotics and 2% charcoal-stripped FBS). After a further 24 h, serum was withdrawn and the cells used in experiments after a further 18–24 h.

Quantification of Na⁺ transport

Snapwell membranes bearing confluent cells (see above) were mounted in Ussing chambers and bathed with bicarbonate-buffered physiological salt solution (composition in mM: NaCl, 112; NaHCO₃, 25; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5 and D-glucose, 11.6, pH 7.3–7.5 when bubbled with 5% CO₂). All cells were maintained under open circuit conditions and transepithelial potential difference (V_t) was monitored (DVC-1000 Voltage/Current Clamp, World Precision Instruments, Stevenage, UK) and recorded (4 Hz) directly to computer disk (ADI Powerlab Interface and associated software; ADI Instruments, Chalgrove, Oxfordshire, UK). Experiments were initiated once V_t had stabilized (40–50 min) and, during each recording, standard pulses of transepithelial current (20 s, –10 μ A·cm⁻²) were

injected every 40 s. The magnitudes of the resultant deflections in V_t (ΔV_t) were then used to calculate transepithelial resistance (R_t) which allowed the equivalent short circuit current (I_{eq} ; i.e. the current that would be required to hold V_t at 0 mV) to be calculated using the expression $I_{eq} = V_t/R_t$. Such calculations were undertaken using spreadsheet software (Microsoft Excel) and, because all experimental manoeuvres were carefully timed, we were able to align the individual data sets, which allowed us to calculate mean values showing the pooled data for each series of experiments undertaken.

All values of V_t are shown relative to an earth electrode in the basolateral bath, which implies that the current carried by cations moving from the lumen to the interstitium will be negative. Such currents are therefore shown as downward deflections of the traces. While this convention differs from that used in many previous electrometric studies of cultured epithelia (see e.g. Wilson *et al.*, 2010), it does accord with more general conventions that are invariably used in electrophysiological studies of single cells. Moreover, the experimental approach used in this study differs from that followed in our earlier studies because, until now, we have always measured short circuit current (I_{sc}) directly from cultures held under voltage clamp (see e.g. Wilson *et al.*, 2010). The values of I_{eq} reported here are, however, very similar to our recently reported values (Wilson *et al.*, 2010) and it is therefore clear that the two approaches do provide essentially identical data. We believe that the present method is preferable because, even in hormone-deprived cells, V_t is -20 to -40 mV and this potential can hyperpolarize to -70 mV during insulin stimulation. Holding V_t at 0 mV in order to measure I_{sc} directly would therefore hyperpolarize the apical membrane potential and establish electrochemical driving forces for ionic movements that may not occur *in vivo*. This has the potential to alter the physiological properties of the cells.

Western analysis of extracted protein

Cells on Transwell membranes were washed with ice-cold phosphate buffered saline 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 then reduced and denatured by

heating (95°C, 5 min) in the presence of 5% β -mercapto-ethanol and 10% sodium dodecyl sulphate (SDS) and fractionated on 10% SDS-polyacrylamide gels. The fractionated proteins were then transferred to Hybond-P membranes (Amersham, Bucks., UK) that were probed using the antibodies described below. Immunoreactive proteins were visualized by enhanced chemiluminescence.

Activity of endogenous kinase activity

PI3K activation was monitored using Western analysis (see above) to monitor changes in the cellular abundance of Ser⁴⁷³-phosphorylated PKB, as it is now clear that the phosphorylation status of this residue provides a reliable read out of cellular PI3K activity (Bayascas and Alessi, 2005). These analyses were undertaken using antibodies against Ser⁴⁷³-phosphorylated and total PKB. Serum and glucocorticoid-inducible kinase 1 (SGK1) activity was monitored by assaying the phosphorylation status of residues (Thr^{346/356/366}) within NDRG1 that are physiological substrates for SGK1 and not for other, closely related kinases including PKB (Murray *et al.*, 2005a; García-Martínez and Alessi, 2008; Inglis *et al.*, 2009). Similarly, the phosphorylation of PRAS40-Ser²⁴⁶ was monitored as an indicator of PKB activity (Kovacina *et al.*, 2003), while P70-S6K-Thr³⁸⁹ phosphorylation was assumed to indicate kinase activity of the target of rapamycin signalling complex 1 (TORC1). The results of all such experiments were quantified by densitometry using the Syngene Genegenius image capturing system, GeneSnap image capturing program and GeneTools densitometry analysis program (SynGene Ltd., Cambridge, UK).

Experimental design and data analysis

All experiments were undertaken using strictly paired protocols in which control and experimental cells were age-matched and at identical passage. Care was taken to ensure that the control and experimental cells were handled identically and electrometric studies were therefore undertaken using parallel Ussing chamber systems, so that we could simultaneously record currents from control and experimental cells. Analyses of extracted proteins were similarly undertaken using paired experimental designs in which the phosphorylation of endogenous proteins was assayed both in hormone-deprived and insulin-stimulated cells. All experiments involving putative inhibitors of protein kinases involved the analysis of protein extracted from pairs of hormone-deprived/insulin-stimulated cells that had either been exposed to the test compound or to the solvent vehicle (0.1% DMSO). Initial experiments showed that this concentration

of DMSO had no effect on any of the parameters studied (not shown). All data are presented as mean \pm SEM, and values of n refer to the number of times a protocol was repeated using cells at different passage. The statistical significance of differences between data derived from hormone-deprived and insulin-stimulated cells were assessed using Student's paired t -test, whereas the results of experiments undertaken using more complex protocols were analysed by one- or two-way analysis of variance/Bonferroni *post hoc* test.

Materials

Amiloride, 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), benzamil, insulin, culture reagents and all general laboratory reagents were from Sigma (Poole, UK) while PI103, rapamycin and inhibitor of Akt (PKB) 1/2 (Akti-1/2, Barnett *et al.*, 2005; Lindsley *et al.*, 2005) were from Merck (Beeston, UK). GSK650394A and GDC-0941 were a generous gift from Prof D.R. Alessi, who had arranged for these compounds to be synthesized within the MRC Protein Phosphorylation Unit (MRC-PPU) at the University of Dundee. Antibodies against Ser⁴⁷³-phosphorylated and total protein kinase B (PKB), and Thr³⁸⁹-phosphorylated and total 70 kDa ribosomal S6 kinase (P70-S6K) were from Upstate (Watford, UK) while the antibodies against Thr^{346/356/366}-phosphorylated and full length forms of the protein encoded by the n-myc downstream regulated gene 1 (NDRG1), and the Ser²⁴⁶-phosphorylated and total forms of the proline-rich 40 kDa substrate of Akt (PKB) (PRAS40) were prepared within the antibody production unit within the MRC-PPU. We are grateful to Prof Sir Phillip Cohen (MRC-PPU) for allowing us access to these antibodies.

Results

Bioelectric properties of hormone-deprived cells

Initial studies of confluent cells ($n = 8$) showed that V_t , R_t and I_{Eq} were normally $\sim -43.8 \pm 1.5$ mV, $\sim 2.5 \pm 0.2$ k Ω ·cm² and $\sim -16.2 \pm 1.7$ μ A·cm⁻², respectively, and, as anticipated (see Bens *et al.*, 1999), amiloride (10 μ M) caused a rapid (5–10 s) and almost complete depolarization of V_t (-3.9 ± 0.4 mV, $P < 0.001$). As this response was accompanied by an increase in R_t (4.4 ± 0.4 k Ω ·cm², $P < 0.001$), this ENaC blocker essentially abolished I_{Eq} (-0.8 ± 0.1 μ A·cm⁻², $P < 0.001$). Further experiments ($n = 9$) in which the apical concentration of amiloride was increased progressively showed that these effects were concentration-dependent and

established that concentrations >10 μ M were maximally effective. The concentration needed for half maximal inhibition (IC₅₀) of I_{Eq} was 0.74 ± 0.01 μ M. Benzamil reproduced these effects of amiloride fully but was ~ 35 -fold more potent (EC₅₀ = 22.7 ± 1 nM, $n = 9$) and, while EIPA also depolarized V_t and increased R_t , the highest concentration tested (3×10^{-4} M) caused only $\sim 75\%$ inhibition of I_{Eq} which made it difficult to estimate IC₅₀ accurately. EIPA was, however, ~ 100 -fold less potent than amiloride (IC₅₀ = 90 ± 20 μ M, $n = 7$). The rank order of potency among these compounds is therefore benzamil $>$ amiloride $>$ EIPA. This observation confirms (see Bens *et al.*, 1999) that hormone-deprived mpkCCD cells spontaneously absorb Na⁺ from the apical bath via an ENaC-dependent mechanism.

Bioelectric response to insulin

Figure 1 shows the results of experiments that explored the effects of insulin on the bioelectric properties of these cells. V_t was ~ -50 mV at the onset of these experiments and, because R_t was ~ 2 k Ω ·cm², these data confirm (see above) that I_{Eq} is normally ~ -20 μ A·cm⁻² (Figure 1). The control data show that V_t tended to depolarize slightly with time and, as R_t was stable, this effect is reflected by a slight decline in I_{Eq} (Figure 1). Insulin (20 nM, basolateral) hyperpolarized V_t to ~ -60 mV and this response became apparent after 3–5 min latency and reached a plateau after ~ 45 min (Figure 1). This hyperpolarization was accompanied by only a small fall in R_t (Figure 1) and further analysis showed that insulin evoked an augmentation of I_{Eq} that reached a plateau after ~ 30 min (Figure 1). Apical amiloride (10 μ M) abolished V_t and increased R_t in unstimulated and insulin-stimulated cells and, even after stimulation with insulin, only negligible currents persisted in the presence of amiloride (Figure 1). The insulin-induced augmentation of I_{Eq} must therefore reflect stimulation of ENaC-mediated Na⁺ absorption. Interestingly, insulin also increased the value of R_t measured in the presence of amiloride (Figure 1), indicating that this hormone must have other effects on these cells (e.g. increasing paracellular resistance). The physiological basis of this action was not investigated.

Insulin-induced phosphorylation of endogenous proteins

Insulin (20 nM, basolateral) increased the cellular abundance of the Ser⁴⁷³-phosphorylated PKB without altering the overall abundance of this protein (Figure 2A,B) and this result shows that insulin evokes PKB-Ser⁴⁷³ phosphorylation. This response reached a maximum after 15–30 min and, although there was some decline from this peak

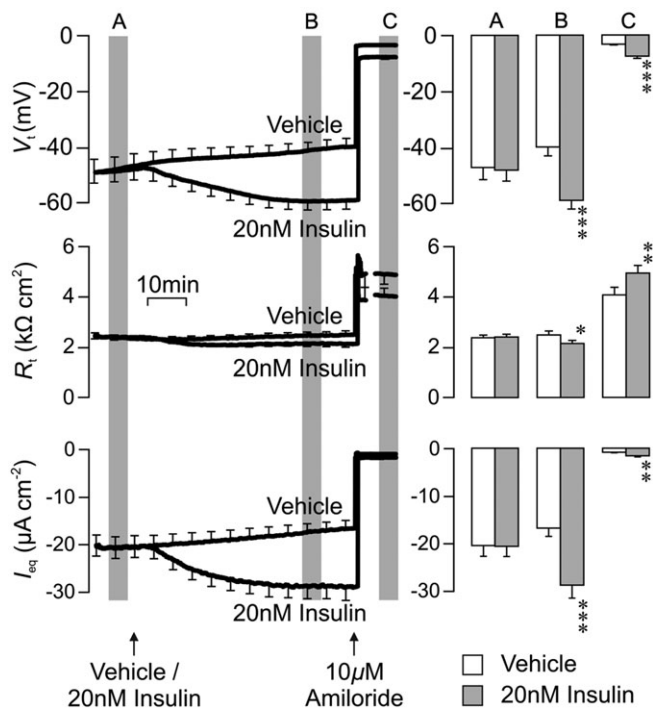


Figure 1

The electrometric response to insulin. Cells grown to confluence on Snapwell membranes were mounted in Ussing chambers so that V_t (upper panels), R_t (middle panels) and I_{eq} (lower panels) could be recorded (see Methods). Because these experiments ($n = 8$) were all undertaken using two parallel Ussing chamber systems, data were recorded simultaneously from pairs of age-matched cultures at identical passage. After an initial equilibration period, the cells were exposed to basolateral insulin (20 nM) or to solvent vehicle (arrow) and amiloride (10 μ M) was then added to the apical bath after a further 60 min had elapsed. The right hand panels all show the mean values of V_t , R_t and I_{eq} measured during (A) the initial equilibration period; (B) once the response to insulin was fully established; and (C) finally after the cells had stabilized in the presence of amiloride. These sampling periods are indicated by the shaded bars. All data are mean \pm SEM and asterisks denote statistically significant (Student's paired t -test) effects of insulin stimulation (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$).

value, increased phosphorylation of PKB-Ser⁴⁷³ persisted for at least 6 h (Figure 2A,B). As the phosphorylation of this residue is dependent upon PI3K (Bayascas and Alessi, 2005), this result shows that insulin normally activates this phospholipid kinase (see review, Cohen, 2006). Insulin (20 nM, basolateral) also increased the abundance of Thr^{346/356/366}-phosphorylated NDRG1 and this response, in common with the phosphorylation of PKB-Ser⁴⁷³, occurred with no change in the overall expression of this protein. The insulin-induced phosphorylation of these residues followed a very similar time course to the phosphorylation of PKB-Ser⁴⁷³ (Figure 2C,D) and, because NDRG1-Thr^{346/356/366} phosphorylation is catalyzed by SGK1 and not by other related kinases, including PKB (Murray *et al.*, 2005a,b), this

result shows that insulin also activates SGK1 (Cohen, 2006). As insulin had no effect upon the overall abundance of PKB or NDRG1 (Figure 2A–D), in all subsequent experiments changes to the cellular abundance of the Ser⁴⁷³- and Thr^{346/356/366}-phosphorylated forms of these proteins were assumed to be a reliable biomarker of increased phosphorylation of these residues. All such data were normalized to the phosphoprotein abundances measured in hormone-deprived cells. We did, however, continue to monitor the overall expression of PKB and NDRG1 in all experiments (data not shown).

PI3K-mediated phosphorylation of PKB-Ser⁴⁷³ is an important part of the mechanism that allows hormones to activate this protein kinase (Bayascas and Alessi, 2005; Cohen, 2006); therefore, we also explored the effects of insulin on the phosphorylation of PRAS40-Ser²⁴⁶, an endogenous PKB substrate (Kovacina *et al.*, 2003). Analysis of the data derived from these experiments showed that insulin (20 nM, 30 min) did increase the abundance of Ser²⁴⁶-phosphorylated PRAS40 (Figure 2E,F) but also established that this response coincided with a small (~20%) fall in the overall expression of PRAS40 (Figure 2E,G). It is therefore possible that the phosphorylation of PRAS40-Ser²⁴⁶ targets this protein for degradation. However, in the present context, the most significant result of this observation is that it implies that changes to the abundance of Ser²⁴⁶-phosphorylated PRAS40 will tend to underestimate the phosphorylation of this residue. We therefore further analyzed these data by normalizing the measured abundance of Ser²⁴⁶-phosphorylated PRAS40 to the corresponding values of overall abundance in order to obtain an indicator of PRAS40-Ser²⁴⁶ phosphorylation. This analysis (Figure 2G), which was used in all subsequent studies, showed that insulin stimulates PRAS40-Ser²⁴⁶ phosphorylation, indicating that it does activate PKB (Kovacina *et al.*, 2003; Cohen, 2006).

Biophysical effects of PI3K inhibitors

Figure 3A shows the results of a series of experiments that used a strictly paired experimental design (see Methods) to explore the effects of wortmannin on the electrometric response to insulin. An examination of the control data indicated that V_t tended to depolarize slightly during the first 30 min of the experiment (initial: -57.0 ± 3.4 mV; 30 min: -50.2 ± 2.1 mV, $P < 0.05$) and, as R_t was stable (initial: 2.1 ± 0.2 k Ω ·cm²; 30 min: 2.2 ± 0.2 k Ω ·cm²), this led to an apparently spontaneous (~15%) decline in I_{eq} (Figure 3A). However, despite this effect, wortmannin consistently inhibited I_{eq} and, after ~30 min exposure to this substance, this

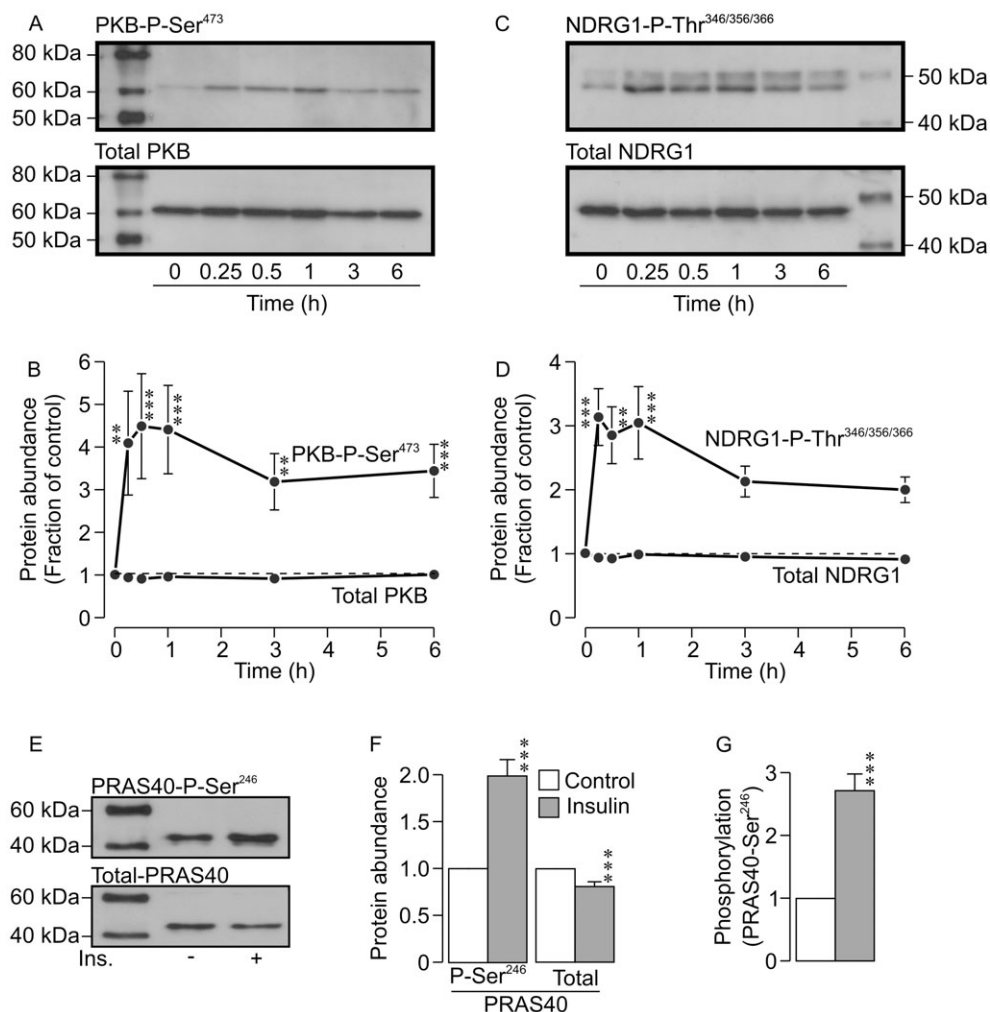


Figure 2

Insulin-evoked phosphorylation of protein kinase B (PKB) Ser⁴⁷³, n-myc downstream regulated gene 1 (NDRG1) Thr^{346/356/366} and 40 kDa proline-rich substrate of Akt (PKB) (PRAS40) Ser²⁴⁶. (A) Typical Western blots showing the effects of insulin (20 nM, 0–6 h) on the cellular abundance of Ser⁴⁷³-phosphorylated and total PKB. (B) Densitometric analysis showing the pooled data from five such experiments. (C) Western blots showing the effects of 20 nM insulin (0–6 h) on the cellular abundance of Thr^{346/356/366}-phosphorylated and total NDRG1. (D) Densitometric analysis showing the pooled data from five such experiments. All data are mean \pm SEM. (E) Typical Western blots showing the effects of insulin (20 nM, 30 min) on the cellular abundance of Ser²⁴⁶-phosphorylated and total PRAS40. (F) Densitometric analysis showing the pooled data from a total of 28 such experiments. (G) Further analysis of these data in which the measured abundance of Ser²⁴⁶-phosphorylated PRAS40 was normalized to the overall abundance of this protein. Asterisks denote statistically significant effects of insulin (** $P < 0.002$, *** $P < 0.001$) determined by one-way ANOVA Bonferroni *post hoc* test (B and D) or Student's paired *t*-test (F and G).

current had decayed to $50.7 \pm 2.7\%$ ($P < 0.001$) of the corresponding control value (Figure 3A). Wortmannin had no effect on R_t over this initial period (initial: $2.0 \pm 0.2 \text{ k}\Omega\cdot\text{cm}^2$; 30 min: $2.2 \pm 0.2 \text{ k}\Omega\cdot\text{cm}^2$) and this suppression of basal current was therefore due to a depolarization of V_t (initial: $-52.6 \pm 4.9 \text{ mV}$; 30 min: $-21.8 \pm 2.4 \text{ mV}$, $P < 0.001$). While the control data confirmed that insulin normally enhances I_{Eq} (Figure 3A) by hyperpolarizing V_t with only a very small effect on R_t , insulin had no effect upon I_{Eq} in wortmannin-treated cells (Figure 3A). It is therefore clear that this inhibitor of PI3K abolished the electrometric response to insulin.

However, analysis of the raw data recorded from wortmannin-treated cells showed that V_t and R_t declined substantially during exposure to insulin so that, after 60 min exposure to this hormone, these parameters had decayed to $-3.6 \pm 2.0 \text{ mV}$ ($P < 0.001$) and $0.5 \pm 0.2 \text{ k}\Omega\cdot\text{cm}^2$ ($P < 0.001$) respectively. In contrast, R_t and V_t were normally stable, as the values measured in control cells that had been exposed to insulin for 60 min were $2.2 \pm 0.1 \text{ k}\Omega\cdot\text{cm}^2$ and $-54.3 \pm 5.3 \text{ mV}$ respectively. At the end of the experiments all cells were exposed to apical amiloride ($10 \mu\text{M}$), normally this reduced I_{Eq} to $-1.4 \pm 0.1 \mu\text{A}\cdot\text{cm}^{-2}$ (>95% inhibition, $P < 0.001$,

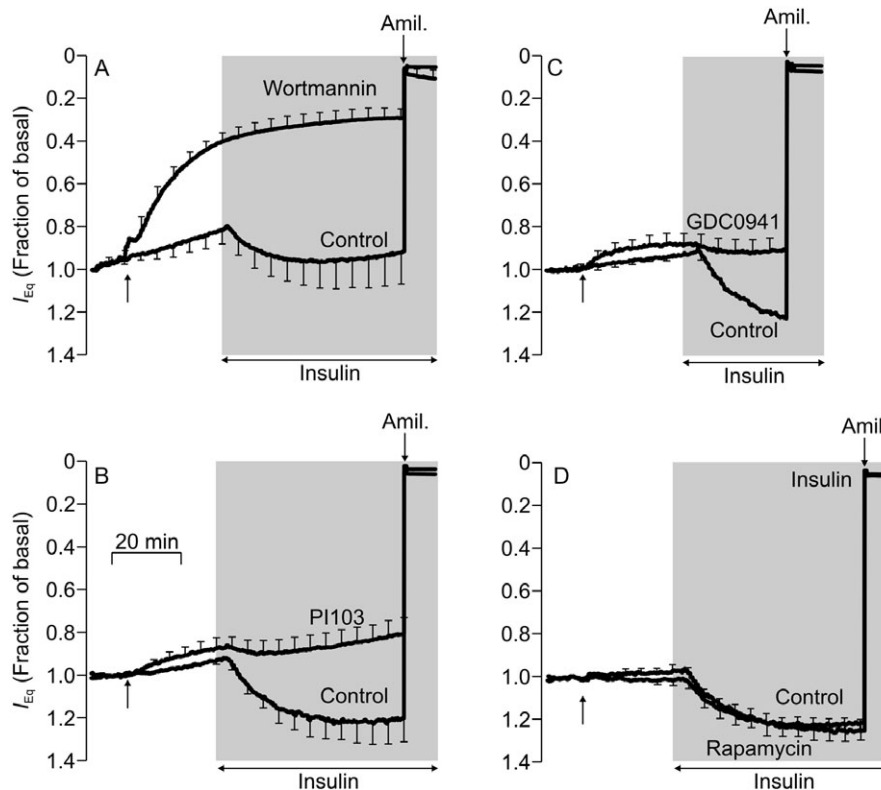


Figure 3

Effects of kinase inhibitors on I_{Eq} . In each experiment, V_t and R_t were recorded simultaneously from pairs of age-matched cells at identical passage and the arrows indicate the addition of either the appropriate test substances or solvent vehicle (control) to the apical and basolateral baths. After 30 min, cells were exposed to insulin (20 nM, basolateral, shaded areas) and experiments were finally terminated by adding 10 μ M amiloride to the apical bath (right hand arrows, Amil). Once recordings were complete, values of I_{Eq} were calculated, normalized to the magnitude of the basal current measured at the onset of the experiment and presented as mean \pm SEM. (A) Effects of wortmannin (0.1 μ M); basal I_{Eq} (control): $-27.5 \pm 2.7 \mu\text{A}\cdot\text{cm}^{-2}$; basal I_{Eq} (test): $-25.4 \pm 3.6 \mu\text{A}\cdot\text{cm}^{-2}$; ($n = 5$). (B) Effects of PI103 (1 μ M); basal I_{Eq} (control): $-26.1 \pm 5.0 \mu\text{A}\cdot\text{cm}^{-2}$; basal I_{Eq} (test): $-30.0 \pm 6.1 \mu\text{A}\cdot\text{cm}^{-2}$; ($n = 6$). (C) Effects of GDC-0941 (1 μ M); basal I_{Eq} (control): $-20.7 \pm 1.3 \mu\text{A}\cdot\text{cm}^{-2}$; basal I_{Eq} (test): $-20.9 \pm 3.3 \mu\text{A}\cdot\text{cm}^{-2}$; ($n = 5$). (D) Effects of rapamycin (0.1 μ M); basal I_{Eq} (control): $-27.8 \pm 3.4 \mu\text{A}\cdot\text{cm}^{-2}$; basal I_{Eq} (test): $-23.3 \pm 4.1 \mu\text{A}\cdot\text{cm}^{-2}$; ($n = 5$).

Figure 3A) and increased R_t to $3.9 \pm 0.6 \text{ k}\Omega\cdot\text{cm}^2$ ($P < 0.001$). However, although amiloride abolished the small current that persisted in the presence of wortmannin (Figure 3A), this inhibition occurred with no increase in R_t ($0.4 \pm 0.2 \text{ k}\Omega\cdot\text{cm}^2$). Wortmannin therefore appears to cause loss of epithelial integrity.

Figure 3B,C show data from experiments in which the same protocol was used to explore the effects of PI103 and GDC-0941. Once again, the control data confirm that hormone-deprived cells generate inwardly directed I_{Eq} and show that insulin normally increases the magnitude of this current (Figure 3B,C). An examination of the underlying data showed that this control response was due to a hyperpolarization of V_t that was associated with a small fall in R_t . Although PI103 and GDC-0941 did cause slight inhibition of I_{Eq} (Figure 3B,C), the currents measured after 30 min exposure to these compounds did not differ significantly from control (PI103: $94.3 \pm 5.0\%$ of control; GDC-0941: $94.6 \pm 5.1\%$ of control) and the corresponding values of R_t

(PI103: $2.1 \pm 0.04 \text{ k}\Omega\cdot\text{cm}^2$; GDC-0941: $2.4 \pm 0.2 \text{ k}\Omega\cdot\text{cm}^2$) were also essentially identical to control ($2.1 \pm 0.2 \text{ k}\Omega\cdot\text{cm}^2$ and $2.3 \pm 0.2 \text{ k}\Omega\cdot\text{cm}^2$ respectively). These compounds, in contrast to wortmannin, therefore had only very small effects on the electrical properties of hormone-deprived cells. Although insulin slightly increased the magnitude of I_{Eq} in cells that had been treated with PI103 (Figure 3B) or GDC-0941 (Figure 3C), the magnitude of these responses were $<10\%$ of control. Moreover, the values of R_t measured at the end of these experiments were also similar to control (not shown) and so, although PI103 and GDC-0941 have almost no effect upon the basal I_{Eq} , they inhibit ($\sim 90\%$) the response to insulin without affecting epithelial integrity.

Biophysical effects of rapamycin

Although often referred to as PI3K inhibitors, wortmannin (Brunn *et al.*, 1993) and PI103 (Raynaud *et al.*, 2007) both inhibit TORC1 and we therefore

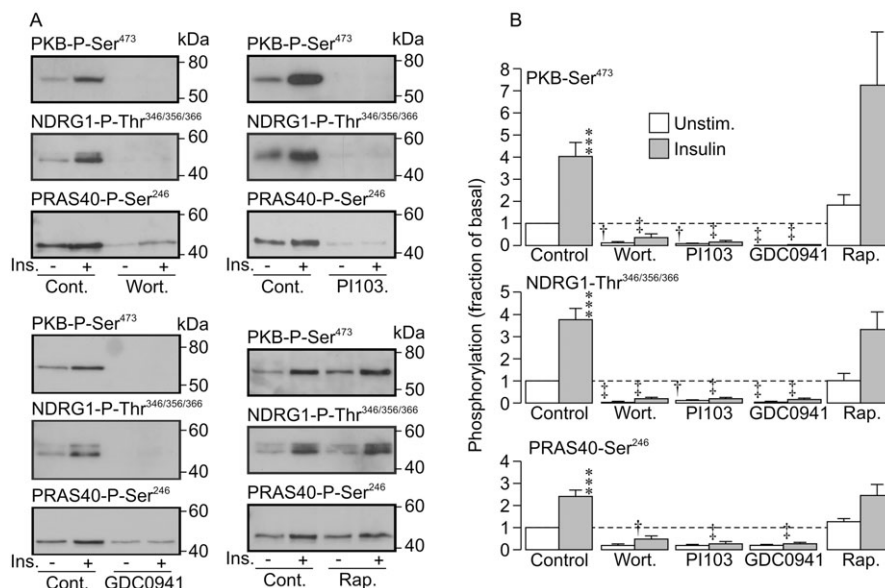


Figure 4

Effects of kinase inhibitors on the phosphorylation of endogenous proteins. (A) Each panel shows the result of an experiment in which age-matched cells at identical passage were either maintained under control conditions (i.e. exposed to solvent vehicle) or exposed to 0.1 μ M wortmannin, 1 μ M PI103, 1 μ M GDC-0941 or 0.1 μ M rapamycin (30 min pre-incubation). Control and inhibitor-treated cells were then incubated for a further 30 min either in hormone-free medium or in medium supplemented with 20 nM insulin (basolateral). Aliquots (40 μ g) of extracted protein were then subjected to Western analysis using antibodies against the Ser⁴⁷³-phosphorylated form of protein kinase B (PKB-Ser⁴⁷³, upper panels), the Thr^{346/356/366}-phosphorylated form of the n-myc downstream regulated gene 1-encoded protein (NDRG1-Thr^{346/356/366}, middle panels) and the Ser²⁴⁶-phosphorylated form of the 40 kDa proline rich Akt (PKB) substrate (PRAS40-Ser²⁴⁶, lower panels). (B) Densitometric analysis showing the pooled data derived from all such experiments ($n = 6$ for each). Data are mean \pm SEM and asterisks denote statistically significant effects of insulin on the phosphorylation of these substrates (* $P < 0.05$, *** $P < 0.001$, Student's paired t -test), while daggers denote statistically significant effects of the inhibitors on the phosphorylation of each substrate under hormone-free conditions and insulin-stimulated conditions ($\dagger P < 0.05$, $\ddagger P < 0.01$; one-way ANOVA/Bonferroni *post hoc* test).

also explored the effects of rapamycin (0.1 μ M, 30 min), a selective inhibitor of this signalling complex (Bain *et al.*, 2007), to ensure that effects on TORC1 did not underlie any of the effects reported here. Rapamycin had no effect upon the I_{eq} in hormone-deprived cells and insulin-stimulated cells (Figure 3D) and also had no effect upon V_t and R_t (not shown).

Effects of PI3K-inhibitors on the phosphorylation of endogenous proteins

The data in Figure 4 confirm that insulin (20 nM, 30 min) normally evokes phosphorylation of PKB-Ser⁴⁷³, NDRG1-Thr^{346/356/366} and PRAS40-Ser²⁴⁶ and show that wortmannin, PI103 and GDC-0941 caused essentially complete dephosphorylation of these residues in both hormone-deprived and insulin-stimulated cells. Because the phosphorylation of this residue is clearly dependent upon PI3K (Bayas-cas and Alessi, 2005), this result shows that all three compounds essentially cause complete inhibition of this regulatory kinase.

Effects of rapamycin on the phosphorylation of endogenous proteins

Rapamycin did not alter the phosphorylation of PKB-Ser⁴⁷³, NDRG1-Thr^{346/356/366} and PRAS40-Ser²⁴⁶ in hormone-deprived or insulin-stimulated cells (Figure 4). Additional experiments explored the effects of rapamycin on the phosphorylation of a residue (P70-S6K-Thr³⁸⁹) that has been identified as a TORC1 substrate (Proud, 2007). These studies ($n = 4$) revealed basal phosphorylation of P70-S6K-Thr³⁸⁹ in hormone-deprived cells and, as anticipated, insulin (20 nM, 30 min) increased the abundance of the Thr³⁸⁹-phosphorylated S6K but had no effect on overall expression. Insulin therefore activates TORC1 in these cells (Proud, 2007). Rapamycin caused essentially complete dephosphorylation of P70-S6K-Thr³⁸⁹ in hormone-deprived and insulin-stimulated cells, indicating that this compound fully inactivates TORC1 (data not shown).

Electrometric effects of GSK650394A

Experiments in which hormone-deprived cells were acutely exposed to GSK650394A (1–10 μ M, added to

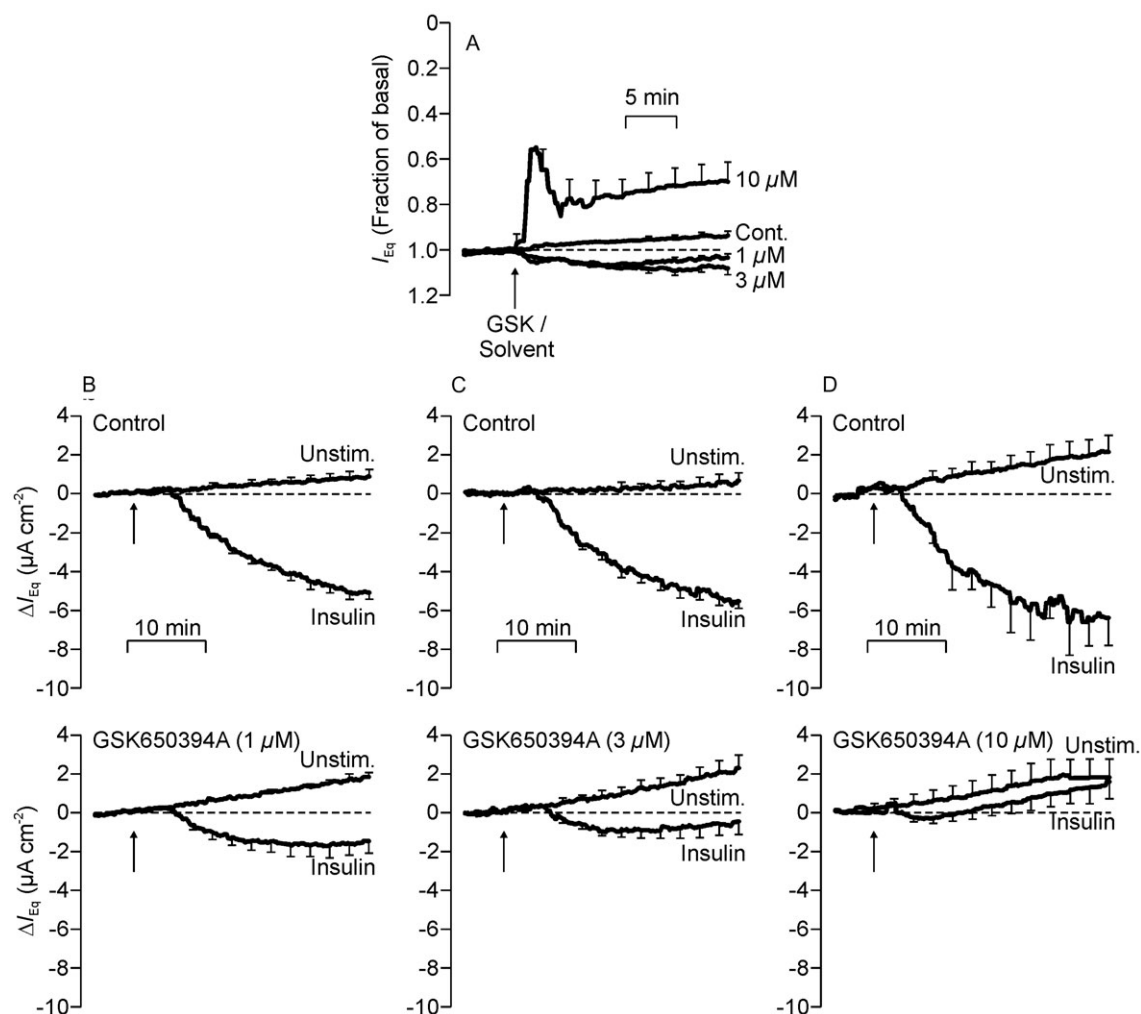


Figure 5

Effects of GSK650394A (GSK) upon the current recorded from hormone-deprived (A) and insulin-stimulated (B–D) cells. (A) Currents ($n = 5$) were initially recorded from hormone-deprived cells under standard conditions and all data were then normalized to the magnitude of the basal current (control: $-16.8 \pm 1.8 \mu\text{A}\cdot\text{cm}^{-2}$; $1 \mu\text{M}$ GSK: $-12.2 \pm 2.1 \mu\text{A}\cdot\text{cm}^{-2}$; $3 \mu\text{M}$ GSK: $-18.1 \pm 3.1 \mu\text{A}\cdot\text{cm}^{-2}$; $10 \mu\text{M}$ GSK: $-21.0 \pm 3.7 \mu\text{A}\cdot\text{cm}^{-2}$) measured before GSK650394A ($1\text{--}10 \mu\text{M}$) or solvent vehicle (control) was added as indicated by the arrow. (B–D) The upper part of each panel shows control data derived from unstimulated and insulin-stimulated (20 nM , arrow) cells while the lower part shows equivalent data derived from age-matched cells at identical passage that had been pre-treated with $1 \mu\text{M}$ (B), $3 \mu\text{M}$ (C) or $10 \mu\text{M}$ (D) GSK650394A (30 min pre-incubation, $n = 6$). These data are shown as change in I_{Eq} from the value measured in the first 5 min of the experimental period (ΔI_{Eq} , mean \pm SEM).

the apical and basolateral baths), an inhibitor of SGK1 (Sherk *et al.*, 2008), showed that this compound had no significant effect upon the I_{Eq} when used at $1 \mu\text{M}$ and $3 \mu\text{M}$ (Figure 5A). However, when used at $10 \mu\text{M}$, GSK650394A rapidly ($2\text{--}3 \text{ min}$) reduced I_{Eq} to a value that was $\sim 60\%$ of the initial, control value ($P < 0.02$, Figure 5A). However, this effect was transient because I_{Eq} subsequently recovered to a plateau value that was $\sim 70\%$ ($P < 0.05$) of that recorded at the onset of the experiment (Figure 5A). Figure 5B–C shows the results of experiments that explored the effects of GSK650394A upon the electrometric response to insulin. These experiments were undertaken using a very strictly

paired experimental design in order to ensure that variability between cells at different passage number did not confound data analysis. Each such experiment therefore involved simultaneously recording I_{Eq} from four confluent cultures so that we could monitor spontaneously developing and insulin-evoked changes in I_{Eq} in both control and GSK650394A-treated cells. Responses to insulin were quantified by subtracting the change in I_{Eq} measured in control cells from the change that developed during exposure to insulin and this, in turn, allowed the effects of GSK650394A to be quantified. Data obtained in this way confirm that insulin normally enhances I_{Eq} and, while this

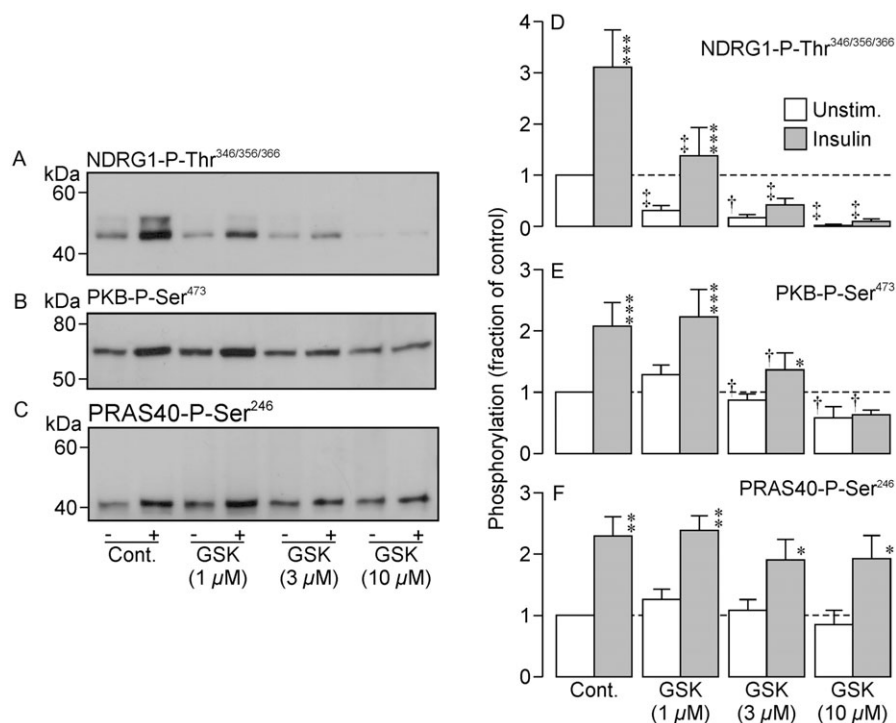


Figure 6

Effects of GSK650394A (GSK) on the phosphorylation of the protein encoded by n-myc downstream regulated gene 1 (NDRG1-Thr^{346/356/366}), protein kinase B (PKB)-Ser⁴⁷³ and the 40 kDa proline-rich substrate of Akt (PKB) (PRAS40)-Ser²⁴⁶. Age-matched cells at identical passage were either maintained in hormone-free medium (Unstim) or exposed to insulin (20 nM, 30 min) under control conditions or in the presence of GSK650394A (GSK, 1 μM, 3 μM or 10 μM, 30 min pre-incubation). Aliquots of extracted protein (40 μg) were subjected to Western analysis using antibodies against the Thr^{346/356/366}-phosphorylated form of NDRG1 (A, D), the Ser⁴⁷³-phosphorylated form of PKB (B, E) or the Ser²⁴⁶-phosphorylated form of PRAS40 (C, F). The left hand panels (A–C) show typical blots illustrating the effects of GSK650394A on the phosphorylation of these residues while the densitometric analysis presented in the right hand panels (D–F) shows pooled data (mean ± SEM) derived from the entire series of experiments ($n = 6$ for each). Asterisks denote statistically significant effects of insulin on the phosphorylation of NDRG1-Thr^{346/356/366} (* $P < 0.05$, ** $P < 0.02$, *** $P < 0.001$, Student's paired t -test), while daggers denote statistically significant effects of GSK650394A on the phosphorylation of this protein in hormone-deprived or insulin-stimulated cells († $P < 0.05$, ‡ $P < 0.001$, two-way ANOVA/Bonferroni *post hoc* test).

response did persist in the presence of 1 μM (Figure 5B) and 3 μM (Figure 5C) GSK650394A, this substance did cause some inhibition (1 μM: $44.5 \pm 7.6\%$ inhibition, $P < 0.01$; 3 μM: $54.8 \pm 1.5\%$ inhibition, $P < 0.001$). GSK650394A caused essentially complete block of this response ($96.9 \pm 5.6\%$ inhibition, $P < 0.02$) at 10 μM (Figure 5D).

Effects of GSK650394A on the phosphorylation of endogenous proteins

GSK650394A (1–10 μM) had no effect on the overall expression of the NDRG1 protein (not shown) but caused a concentration-dependent decline in NDRG1-Thr^{346/356/366} phosphorylation in hormone-deprived and insulin-stimulated cells, and this effect was essentially complete at 10 μM (Figure 6A,D). GSK650394A (1–10 μM) also had no effect on the overall expression of PKB (not shown) and did not alter the abundance of Ser⁴⁷³-phosphorylated PKB in hormone-deprived cells (Figure 6B). However,

GSK650394A did inhibit the insulin-induced phosphorylation of PKB-Ser⁴⁷³ at 3 μM, and essentially abolished this response at 10 μM (Figure 6B,E) and, because the phosphorylation of PKB-Ser⁴⁷³ is dependent upon PI3K (Bayascas and Alessi, 2005), this finding suggests that GSK650394A may prevent the insulin-induced activation of PI3K. However, despite this effect, GSK650394A (1–10 μM) did not alter the phosphorylation of PRAS40-Ser²⁴⁶ in hormone-deprived cells and did not prevent the insulin-induced phosphorylation of this residue (Figure 6C,F). It thus appears that 10 μM GSK650394A blocks signalling via SGK1 but not PKB (see also Sherk *et al.*, 2008).

Electrometric effects of Akti-1/2

Acutely exposing cells to Akti-1/2 (1–10 μM, apical and basolateral) had no discernible effect upon I_{Eq} at 1 and 3 μM (Figure 7A). However, 10 μM Akti-1/2 caused a small (~25%) but significant ($P < 0.01$)

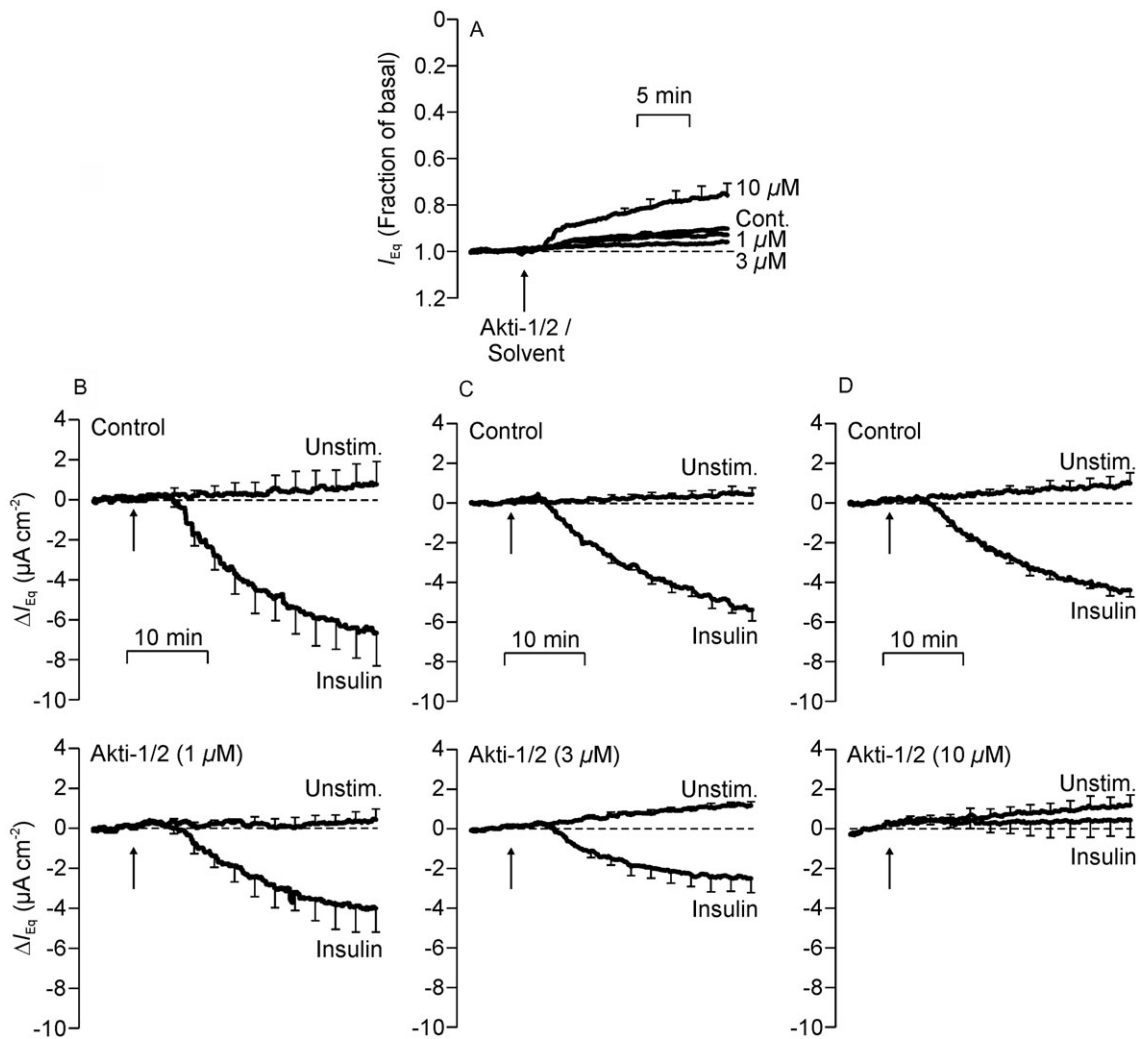


Figure 7

Effects of Akti-1/2 on the current recorded from hormone-deprived (A) and insulin-stimulated cells (B–D). (A) Currents were initially recorded from hormone-deprived cells under standard conditions and all data are normalized to the magnitude of this basal current (control: $-17.8 \pm 1.7 \mu\text{A}\cdot\text{cm}^{-2}$; 1 μM Akti-1/2: $-20.3 \pm 2.0 \mu\text{A}\cdot\text{cm}^{-2}$; 3 μM Akti-1/2: $-15.3 \pm 0.9 \mu\text{A}\cdot\text{cm}^{-2}$; 10 μM Akti-1/2: $-15.1 \pm 3.3 \mu\text{A}\cdot\text{cm}^{-2}$) that was recorded over the 5 min period preceding the addition of Akti-1/2 (1–10 μM) or solvent vehicle (control) as indicated by the arrow ($n = 5$ for each). (B–D) The upper part of each panel shows data derived from unstimulated and insulin-stimulated (20 nM, arrow) control cells while the lower panels show equivalent data derived from age-matched cells at identical passage that had been pre-treated with 1 μM (B), 3 μM (C) or 10 μM (D) Akti-1/2 (30 min pre-incubation, $n = 6$). These data are shown as change in I_{Eq} from the value measured in the first 5 min of the experimental period (ΔI_{Eq} , mean \pm SEM).

inhibition of the basal current that became apparent after a latency period of ~ 2 min and developed over the following 10–15 min (Figure 7A). Figure 7B–D shows the results of experiments that explored the effects of Akti-1/2 (1–10 μM) on the insulin-induced augmentation of I_{Eq} using the method described previously. The control data confirm that insulin consistently increased the magnitude of this current and, although Akti-1/2 appeared to enhance the spontaneous decline in I_{Eq} when used at 3 μM and 10 μM , these effects were not statistically significant. Responses to insulin were clearly seen in cells

exposed to 1 μM (Figure 7B) and 3 μM Akti-1/2 (Figure 7C), but these were smaller than control (1 μM : $43.4 \pm 14.6\%$ inhibition, $P < 0.05$; 3 μM : $31.3 \pm 12.5\%$ inhibition, $P < 0.05$) and this response was essentially abolished ($80.8 \pm 7.3\%$ inhibition, $P < 0.02$) by 10 μM Akti-1/2 (Figure 7D).

Effects of Akti-1/2 on the phosphorylation of endogenous proteins

Exposing cells to Akti-1/2 had no effect on the overall expression of PKB or PRAS40 (not shown)

but did cause a concentration-dependent decline in the abundance of Ser⁴⁷³-phosphorylated PKB (Figure 8A,D) and Ser²⁴⁶-phosphorylated PRAS40 (Figure 8B,E) in both hormone-deprived and insulin-stimulated cells. Indeed, these phosphoproteins were almost undetectable after exposure to 10 μ M Akti-1/2, indicating essentially complete inactivation of PKB. Akti-1/2 also had no discernible effect upon the overall expression of NDRG1 (not shown) and the data in Figure 8C,F therefore show that Akti-1/2 caused concentration-dependent dephosphorylation of NDRG1-Thr^{346/356/366} in hormone-deprived and insulin-stimulated cells. Indeed, 10 μ M Akti-1/2 essentially abolished the basal phosphorylation of NDRG1-Thr^{346/356/366} and the response to insulin (Figure 8C and F) and, because these residues are phosphorylated by SGK1 and not by PKB (Murray *et al.*, 2005a,b), these data show that Akti-1/2 blocks signalling via both PKB and SGK1 under the present conditions.

Discussion

Na⁺ transport in hormone-deprived cells

Hormone-deprived mpkCCD cells absorb Na⁺ from the apical bath via an apparently spontaneous mechanism dependent upon ENaC and it is therefore clear that Na⁺ absorption can occur independently of stimulating hormones, a finding that accords with data from several earlier studies of ASDN-derived cell lines (see e.g. Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; Paunescu *et al.*, 2000; Alvarez De La Rosa and Canessa, 2003). Analyses of extracted protein showed that hormone-deprived cells displayed basal phosphorylation of PKB-Ser⁴⁷³, establishing (Bayascas and Alessi, 2005) that PI3K is active under these conditions. It is therefore interesting that this regulatory kinase seems to contribute to the control of ENaC function. Early evidence of this came from the observation that LY294002, a PI3K inhibitor, blocks Na⁺

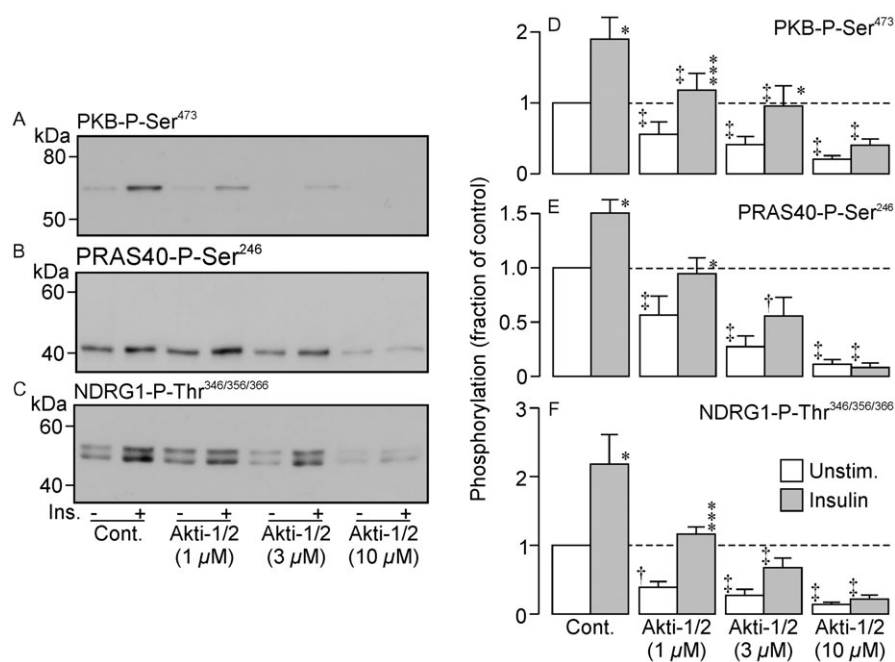


Figure 8

Effects of Akti-1/2 on the phosphorylation of protein kinase B (PKB) -Ser⁴⁷³, the 40 kDa proline-rich substrate of Akt (PKB) (PRAS40) -Ser²⁴⁶ and the protein encoded by n-myc downstream regulated gene 1 (NDRG1-Thr^{346/356/366}). Age-matched cells at identical passage were either maintained in hormone-free medium (Unstim) or exposed to insulin (20 nM, 30 min). Experiments were undertaken using cells that had been exposed to the solvent vehicle (control) or to Akti-1/2 (1 μ M, 3 μ M or 10 μ M, 30 min pre-incubation). All cells were then lysed and aliquots of extracted protein (40 μ g) subjected to Western analysis using antibodies against the Ser⁴⁷³-phosphorylated PKB (A, D), the Ser²⁴⁶-phosphorylated PRAS40 (B, E) or the Thr^{346/356/366}-phosphorylated form of NDRG1 (C, F). The left-hand panels (A–C) show typical blots illustrating the effects of Akti-1/2 on the phosphorylation of these residues, while the densitometric analyses presented in the right-hand panels (D–F) show the pooled data (mean \pm SEM) from the entire series of experiments ($n = 6$). Asterisks denote statistically significant effects of insulin on the phosphorylation of these proteins (* $P < 0.05$, *** $P < 0.001$, Student's paired t -test), while daggers denote statistically significant effects of Akti-1/2 on the basal phosphorylation of each substrate under hormone-free conditions or in the presence of insulin († $P < 0.05$, ‡ $P < 0.001$, two-way ANOVA/Bonferroni *post hoc* test).

absorption in hormone-deprived cells by progressively reducing the number of active Na⁺ channels in the apical membrane (Paunescu *et al.*, 2000). Moreover, structural studies (Schild *et al.*, 1996) showed that each ENaC subunit contains a C-terminal PY motif that provides binding sites for the neural precursor cell expressed, developmentally down-regulated protein 4-2 (Nedd-4/2). The binding of Nedd-4/2 (Kanelis *et al.*, 2001) to these motifs targets the ENaC channel complex for internalization and degradation and such Nedd-4/2-mediated internalization/degradation of ENaC seems to limit the rate of Na⁺ absorption by restricting the Na⁺ conductance of the apical membrane (G_{Na}) (Debonneville *et al.*, 2001; Wang *et al.*, 2001; Snyder *et al.*, 2004). PI3K contributes to this mechanism by maintaining the catalytic activity of SGK1 (Kobayashi and Cohen, 1999; Park *et al.*, 1999), a protein kinase that prevents the interaction between Nedd-4/2 and ENaC by phosphorylating Nedd-4/2 at Ser³⁴² and Ser⁴²⁸ (Debonneville *et al.*, 2001; Wang *et al.*, 2001; Snyder *et al.*, 2004). PI3K inhibitors such as LY294002 are therefore thought to inhibit Na⁺ transport by depriving the cell of SGK1 activity and thus facilitating the Nedd-4/2-mediated internalization/degradation of ENaC. However, although widely used, such small molecule kinase inhibitors almost always affect multiple targets and it is now clear that, as well as inactivating PI3K, LY294002 also inhibits TORC1, PLK1 (polo-like kinase 1), PIMK (provirus integration site for Moloney murine leukaemia virus kinase) 1 and 3, HIPK (homeodomain-interacting protein kinase) 2, GSK3 (glycogen synthase kinase 3) and CK2 (casein kinase 2) (Davies *et al.*, 2000; Bain *et al.*, 2007). LY294002 therefore displays poor selectivity for PI3K and its use to block signalling via this kinase is therefore no longer recommended (Bain *et al.*, 2007). Indeed, the fact that LY294002 inhibits CK2 makes it particularly unsuitable for studies of Na⁺-absorbing epithelia as CK2 does appear to contribute to the control of ENaC activity (Shi *et al.*, 2002; Bachhuber *et al.*, 2008).

The present study therefore explored the effects of wortmannin, PI103 and GDC-0941 as these substances all appear to be relatively selective PI3K inhibitors (Bain *et al.*, 2007; Folkes *et al.*, 2008). The first such studies showed that wortmannin inhibited Na⁺ absorption in hormone-deprived cells and this is consistent with the idea that PI3K is essential for the maintenance of basal Na⁺ absorption (Paunescu *et al.*, 2000). However, PI103 and GDC-0941 had very little effect on basal Na⁺ transport and yet an analysis of extracted protein showed very clearly that wortmannin, PI103 and GDC-0941 all caused essentially complete dephosphorylation of PKB-

Ser⁴⁷³, NDRG1-Thr^{346/356/366} and PRAS40-Ser²⁴⁶. It is therefore clear that all three compounds do inactivate PI3K fully under the present conditions, and also block signalling via SGK1 and PKB, protein kinases that are important downstream targets of PI3K (Kobayashi and Cohen, 1999; Park *et al.*, 1999; Bayascas and Alessi, 2005). The main effect of wortmannin which we now report cannot therefore be attributed to more effective inhibition of PI3K and it is therefore interesting that *in vitro* studies have shown that this compound can also inactivate PLK1 and smooth muscle myosin light chain kinase (SmMLCK). While we are unaware of any data implicating PLK1 in the control of epithelial Na⁺ absorption, it has been suggested that SmMLCK might contribute to the regulation of ENaC trafficking (Tokuda *et al.*, 2002) and, because PI103 does not appear to act in this way (Bain *et al.*, 2007), effects on SmMLCK might explain the effect of wortmannin which we now report.

Our data from cells treated with PI103 and GDC-0941 show that signalling via PI3K/SGK1 does not make a major contribution to Na⁺ absorption in hormone-deprived cells and this contrasts with earlier data (see e.g. Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; Paunescu *et al.*, 2000; Alvarez De La Rosa and Canessa, 2003). Data from recent studies of H441 human airway epithelial cells are interesting in this context. While these cells absorb Na⁺ via an ENaC-dependent mechanism, this absorptive phenotype is seen only in glucocorticoid stimulated cells (Clunes *et al.*, 2004; Ramminger *et al.*, 2004) and we have therefore used this cell type as a model system to explore the factors that allow steroid hormones to regulate G_{Na} . It is abundantly clear that glucocorticoids do activate SGK1 in H441 cells and the fact that the physiological effects of glucocorticoid-stimulation are reproduced by transient expression of a constitutively active form of SGK1 suggest strongly that this kinase is involved in this response (Thomas *et al.*, 2004; Brown *et al.*, 2008; Inglis *et al.*, 2009). However, our data also show that SGK1 is active in hormone-deprived cells, despite the fact that G_{Na} is negligible and it is far from clear why this basal activity of SGK1 is not transduced into a Na⁺-absorbing phenotype (Inglis *et al.*, 2009). Moreover, transient expression of a dominant negative SGK1 mutant suppresses the glucocorticoid-induced activation of the endogenous kinase without blocking the associated rise in G_{Na} and these findings (Inglis *et al.*, 2009), in common with the present data, suggest that signalling via PI3K/SGK1 is not critical to the control of ENaC function.

It is therefore interesting that steroid hormones induce the expression of a number of other proteins

that seem to be involved in the hormonal control of G_{Na} . For example, aldosterone evokes expression of the protein encoded by n-myc downstream regulated gene 2 (NDRG2) and this protein has been shown to increase the activity of ENaC expressed in *Xenopus* oocytes and Fisher rat thyroid cells (Wielpütz *et al.*, 2007). Steroid hormones also induce expression of glucocorticoid-inducible leucine zipper proteins 1-3 (GILZ1-3) and a growing body of evidence implicates these proteins in the control of ENaC function (Soundararajan *et al.*, 2005; 2007). Interestingly, recent work has suggested that GILZ1-3 might act in cooperation with SGK1 (Soundararajan *et al.*, 2009) and established that transient expression of GILZ1 mimics the electrophysiological effects of glucocorticoid stimulation in H441 cells (Watt *et al.*, 2009). It is therefore possible that these mechanisms might contribute to the maintenance of basal Na^+ transport in hormone-deprived mpkCCD cells.

Insulin-induced Na^+ transport

Insulin acutely stimulated Na^+ absorption (see also Wilson *et al.*, 2010) and induced phosphorylation of PKB-Ser⁴⁷³, NDRG1-Thr^{346/356/366} and PRAS40-Ser²⁴⁶, indicating that this hormone activates PI3K and also increases the activity of the downstream protein kinases SGK1 (see also Wilson *et al.*, 2010) and PKB. While we have assumed that these responses to insulin are mediated via true insulin receptors, we cannot exclude the possibility that these effects may be mediated, at least in part, via receptors for insulin-like growth factor 1 (IGF-1), as the concentration of insulin used here (20 nM) may allow activation of these receptors (Gonzalez-Rodriguez *et al.*, 2007). However, IGF-1 and insulin are thought to control Na^+ transport via very similar mechanisms (Gonzalez-Rodriguez *et al.*, 2007) and, while wortmannin, PI103 and GDC-0941 had differing effects upon basal I_{Eq} (see above), these compounds all caused essentially complete inhibition of insulin-induced Na^+ absorption and abolished the insulin-induced phosphorylation of endogenous proteins. While signalling via PI3K/SGK1 does not seem to be important in the maintenance of basal Na^+ absorption (see above), our data suggest strongly that this signalling pathway is critical to insulin-induced Na^+ transport. This finding accords well with a number of earlier studies which indicate that insulin stimulates the trafficking of ENaC subunits to the apical membrane via a PI3K-dependent mechanism (see for example Blazer-Yost *et al.*, 1998; 2003; Record *et al.*, 1998; Wang *et al.*, 2001; Arteaga and Canessa, 2006). Indeed, IGF-1 has recently been shown (Gonzalez-Rodriguez *et al.*, 2007) to cause a

PI3K-dependent increase in the phosphorylation/expression of SGK1 in mouse cortical collecting duct cells. However, this finding is based upon data obtained by probing Western blots with an antibody against total SGK1 and, under these conditions, changes to the phosphorylation status of this protein are inferred by the appearance of multiple, less mobile bands. While it is highly likely that this apparent phosphorylation SGK1 does lead to an increase in catalytic activity (Kobayashi and Cohen, 1999; Park *et al.*, 1999), it is important to stress that such measurements do not provide any information relating to the catalytic activity of SGK1. In contrast, the present study assessed the activity of SGK1 by monitoring the phosphorylation of an endogenous, SGK1 substrate (NDRG1-Thr^{346/356/366}) and this relatively new approach (Murray *et al.*, 2005a; Inglis *et al.*, 2009) allows us to show unequivocally that insulin-induced Na^+ transport is associated with PI3K-dependent activation of SGK1.

Effects of rapamycin

As well as inhibiting PI3K, wortmannin and PI103 also block signalling via TORC1 (Brunn *et al.*, 1993; Raynaud *et al.*, 2007), a kinase activated by insulin that plays an important role in the control of cellular metabolism (Proud, 2007). Because it has been suggested that TORC1 might contribute to the control of SGK1 activity by phosphorylating SGK1-Ser⁴²² (Hong *et al.*, 2008), we also explored the effects of rapamycin, an extremely selective TORC1 inhibitor (Bain *et al.*, 2007). Our data show clearly that rapamycin did not alter the currents generated by hormone-deprived cells; did not modify the electrometric response to insulin, and had no effect upon cellular PI3K, SGK1 and PKB activity in hormone-deprived and insulin-stimulated cells. However, a separate series of experiments confirmed that insulin did evoke P70-S6K-Thr³⁸⁹ phosphorylation, indicating that this hormone does activate TORC1 (Proud, 2007). Moreover, rapamycin caused complete dephosphorylation of P70-S6K-Thr³⁸⁹ in hormone-deprived and insulin-stimulated cells, indicating that this substance fully inactivates TORC1. Our data, in contrast with those presented by Hong *et al.* (2008), therefore provide no evidence to support the idea that TORC1 is involved in the control of SGK1 activity and it is therefore interesting that recently published data suggest that the apparent rapamycin-sensitive phosphorylation of SGK1-Ser⁴²² reported by Hong *et al.* (2008) was actually an artefact caused by the inappropriate use of poorly selective antibodies (García-Martínez and Alessi, 2008).

Physiological basis of insulin-induced Na⁺ absorption

While most evidence suggests that insulin-induced Na⁺ transport reflects PI3K/SGK1-mediated inhibition of Nedd-4/2 (Record *et al.*, 1998; Debonneville *et al.*, 2001; Wang *et al.*, 2001; Snyder *et al.*, 2002; Alvarez De La Rosa and Canessa, 2003), insulin also induces PI3K-dependent activation of PKB (Bayascas and Alessi, 2005; Cohen, 2006). Indeed, it is the activation of PKB that allows insulin to promote glucose uptake by inducing the translocation of the type 4 glucose transporter to the plasma membrane (Cong *et al.*, 1997). It is therefore interesting that studies of Fisher rat thyroid cells heterologously expressing α -, β - and γ -ENaC have suggested that PKB might contribute to the control of G_{Na} by catalyzing the phosphorylation of Nedd-4/2 (Lee *et al.*, 2007). However, despite this seemingly clear result, studies of A6 cells heterologously expressing wild-type and mutant forms of SGK1 (Alvarez De La Rosa and Canessa, 2003) and PKB (Arteaga and Canessa, 2006) indicate that PKB is not involved in the hormonal control of Na⁺ absorption. In an attempt to resolve this apparent contradiction, we also explored the effects of GSK650394A and Akti-1/2, as these compounds have, respectively, been reported to inhibit SGK1 (Sherk *et al.*, 2008) and PKB (Barnett *et al.*, 2005; Lindsley *et al.*, 2005) selectively.

GSK650394A had a relatively modest effect (~30% inhibition) on Na⁺ transport in hormone-deprived cells and caused concentration-dependent inhibition of the electrometric response to insulin with essentially complete block at 10 μ M. Analyses of extracted proteins showed that GSK650394A caused dephosphorylation of NDRG1-Thr^{346/356/366} in both hormone-deprived and insulin-stimulated cells and this effect was also essentially complete at 10 μ M. The highest concentrations of GSK650394A tested did seem to cause some inhibition of insulin-induced PKB-Ser⁴⁷³ phosphorylation, which raised the possibility that GSK650394A may also cause some inhibition of PI3K. However, GSK650394A had no effect on the phosphorylation of PRAS40-Ser²⁴⁶, even at 10 μ M, and it is therefore clear that this substance does not block the insulin-induced phosphorylation of PKB substrates. While this may appear surprising in view of the inhibition of PKB-Ser⁴⁷³ phosphorylation (see above), Logie *et al.* (2007) have shown that there is considerable extra capacity in the PKB-dependent signalling pathway. Indeed, their studies of liver cells indicated that endogenous PKB activity must be reduced to <10% of the basal level before the phosphorylation of downstream targets is compromised (Logie *et al.*, 2007). The present data therefore confirm (Sherk *et al.*, 2008) that GSK650394A suppresses signalling

via SGK1 and not via PKB. The finding that this compound fully suppressed the electrometric response to insulin therefore supports the view that this response is mediated via PI3K/SGK1 (Alvarez De La Rosa and Canessa, 2003; Arteaga and Canessa, 2006) rather than via PI3K/PKB (Lee *et al.*, 2007).

Akti-1/2 also caused concentration-dependent inhibition of the electrometric response to insulin and this effect, in common with the effect of GSK650394A, was essentially complete at 10 μ M. Akti-1/2 also caused essentially complete dephosphorylation of PKB-Ser⁴⁷³ and PRAS40-Ser²⁴⁶ in both hormone-deprived and insulin-stimulated cells, which confirms that this compound is an effective inhibitor of PKB (Barnett *et al.*, 2005; Lindsley *et al.*, 2005). However, our data suggest that 10 μ M Akti-1/2 is needed for complete inhibition of PKB and this contrasts with data from liver cells, which reported full inhibition at concentrations below 1 μ M (Logie *et al.*, 2007). Our findings do, however, accord with earlier work, which suggests that concentrations as high as 20 μ M are needed to inhibit PKB fully (Barnett *et al.*, 2005). The present data show that Akti-1/2 also causes dephosphorylation of NDRG1-Thr^{346/356/366} and this effect, in common with the dephosphorylation of PRAS40-Ser²⁴⁶, was complete at 10 μ M. As the phosphorylation of NDRG1-Thr^{346/356/366} is strictly dependent upon SGK1 (Murray *et al.*, 2005a,b), this result shows that Akti-1/2 inhibits both PKB and SGK1 under the present conditions and it is therefore interesting that 10 μ M Akti-1/2 has been shown to cause substantial (~75%) inhibition of SGK1 *in vitro* (Logie *et al.*, 2007). Hence, our data indicate that Akti-1/2 is not a selective PKB blocker, and this result highlights the difficulties inherent to all experiments based around such small molecule inhibitors of protein kinases (Bain *et al.*, 2007). All data obtained using Akti-1/2 must therefore be treated with caution and, at present, it is impossible for us to exclude the possibility that PKB may contribute to the control of Na⁺ transport by acting in concert with SGK1 (Lee *et al.*, 2007).

Significance of the present findings

The present data suggest that signalling via PI3K/SGK1 is not important to the maintenance of basal Na⁺ transport, because hormone-deprived cells continued to absorb Na⁺ when PI3K had been fully inactivated using PI103 or GDC-0941. It is therefore understandable that deletion of the *sgk1* gene has no overt effect upon renal Na⁺ handling in animals fed a normal diet (Wulff *et al.*, 2002) and this finding, in common with the present data, suggests strongly that SGK1 is not involved in basal Na⁺ transport. However, signalling via PI3K does seem to

be critical for insulin-stimulated Na⁺ transport and the finding that GSK650394A abolished the insulin-induced Na⁺ transport suggests strongly that this response is mediated via SGK1 (see also Record *et al.*, 1998; Wang *et al.*, 2001; Arteaga *et al.*, 2006). Interestingly, *sgk1* gene deletion does block the insulin-induced reduction in urinary Na⁺ excretion (Huang *et al.*, 2006) and it is therefore possible that drugs such as GSK650394A, which selectively inactivate SGK1, might become useful in the treatment of fluid retention/oedema that can complicate the management of type 2 diabetes.

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Conflict of interest

The authors report no conflict of interest.

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