The regulation of selective and nonselective Na⁺ conductances in H441 human airway epithelial cells

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The regulation of selective and nonselective Na⁺ conductances in H441 human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 294: L942–L954, 2008. First published February 29, 2008; doi:10.1152/ajplung.00240.2007.—Analysis of membrane currents recorded from hormone-deprived H441 cells showed that the membrane potential (V_m) in single cells (approximately −80 mV) was unaffected by lowering [Na⁺], or [Cl⁻], indicating that cellular Na⁺ and Cl⁻ conductances (G_{Na} and G_{Cl}, respectively) are negligible. Although insulin (20 nM, −24 h) and dexamethasone (0.2 µM, −24 h) both depolarized V_m by −20 mV, the response to insulin reflected a rise in G_{Cl} mediated via phosphatidylinositol 3-kinase (PI3K) whereas dexamethasone acted by inducing a serum- and glucocorticoid-regulated kinase 1 (SGK1)-dependent rise in G_{Na}. Although insulin stimulation/PI3K-P_110 expression did not directly increase G_{Na}, these maneuvers augmented the dexamethasone-induced conductance. The glucocorticoid/SGK1-induced G_{Na} in single cells discriminated poorly between Na⁺ and K⁺ (P_{Na/P_K} ~0.6), was insensitive to amiloride (1 mM), but was partially blocked by LaCl₃ (La³⁺; 1 mM, ~80%), pimozone (0.1 mM, ~40%), and dichlorobenzamil (15 µM, ~15%). Cells growing as small groups, on the other hand, expressed an amiloride-sensitive (10 µM), selective G_{Na} that displayed the same pattern of hormonal regulation as the nonselective conductance in single cells. These data therefore 1) confirm that H441 cells can express selective or nonselective G_{Na}, 2) show that these conductances are both induced by glucocorticoids/SGK1 and subject to PI3K-dependent regulation, and 3) establish that cell-cell contact is vitally important to the development of Na⁺ selectivity and amiloride sensitivity.

epithelial Na⁺ channel; serum- and glucocorticoid regulated kinase 1; phosphatidylinositol 3-kinase; H441 cells; pulmonary Na⁺ absorption

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incubated (24–48 h) in medium identical to that described above except that the serum components were replaced with FBS (8.5%) that had been dialyzed to remove hormones/growth factors. “Hormone-deprived” cells were maintained under these conditions for 24–48 h before being used in experiments, whereas hormone-treated cells were incubated in medium supplemented with dexamethasone (0.2 μM) or insulin (20 nM) either alone or in combination.

Electrophysiology. Membrane currents (I_m) were recorded using the perforated patch recording technique, and only brief details are presented here since this method is described in detail elsewhere (see Refs. 14, 23, 56). The pipette filling solution contained in mM: NaCl, 10; KCl, 18; potassium gluconate, 92; MgCl_2, 0.5; EGTA, 1; HEPES, 10; and its pH was adjusted to 7.2 with KOH, which brought K^+ to 113.3 mM. The standard bath solution contained in mM: NaCl, 140; KCl, 4.5; MgCl_2, 1; CaCl_2, 2.5; HEPES, 10; glucose, 5; and its pH was adjusted to 7.4 with NaOH, which brought Na^+ to 144.4 mM. Under standard conditions (22°C), the equilibrium potentials for Na^+, K^+, and Cl^- (E_{Na}, E_{K}, and E_{Cl}, respectively) were therefore 67.8 mV, -81.9 mV, and -41.9 mV, respectively. The K^+-rich (134.5 mM) bath solution was prepared by isosmotically replacing the Na^+ with K^+ ([Na^+]_o = 10 mM), whereas the low Cl^- solution ([Cl^-]_o = 26.5 mM) was prepared by isosmotically replacing Cl^- with either gluconate or I^-.

Experimental design and data analysis. Unless otherwise stated, all reported currents were recorded from single cells, although, in some experiments, data were recorded from cells growing in small groups that appeared to contain 3–5 cells. In each experiment, the mean current evoked by driving the holding potential (V_{Hold}) through a series of four ramps (87 mV to -113 mV), each of 4-s duration, was recorded, and plots showing the relationship between I_m and V_{Hold} were constructed. The resting membrane potential (V_m) was inferred from the reversal potential (V_{Rev}; i.e., the value of V_{Hold} at which I_m is 0), whereas total membrane conductance (G_{Tot}) was estimated as the slope of the I_m-V_{Hold} relationship over a physiologically relevant range of potentials (-113 to 0 mV). The input capacitance (C_m) of each preparation was carefully noted, and the magnitudes of all currents and all values of membrane conductance were subsequently normalized to the mean value of C_m associated with a single cell (35 pF). Such data are therefore expressed as picocoulombs or nanomoles per average sized cell (pA per cell or nM per cell). This manipulation was undertaken to ensure that variations between the sizes of different groups did not contribute to the variability in the presented data. The magnitudes of currents recorded from single cells and from small groups of cells can thus be compared directly. Cited values of V_{Hold}, V_m, and V_{Rev} have all been corrected for the liquid junction potential between the bath and pipette solutions (-13 mV), and, since the bath was always grounded via a salt bridge filled with 3 M KCl/4% agar, the solution changes imposed during the present study had insignificant effects (<1 mV) on this potential (6).

Membrane Na^+ current (I_{Na}) was quantified by recording I_m under control conditions and repeating this measurement 20–30 s after the bath Na^+ had been largely ([Na^+]_o = 10 mM) replaced with N-methyl-d-glucammonium (NMDG^+), a nominally impermeant cation. The current that persisted in the presence of NMDG^+ was then subtracted from the corresponding record of total current to isolate I_{Na}. The N-phenylanthranilic acid (usually known as DPC)-sensitive (I_{DPC}), amiloride-sensitive (I_{Ami}), and LaCl_3 (La^3+)-sensitive (I_{La}) components of I_{Na} were derived using directly analogous procedures. G_{Na} was estimated by regression analysis of the relationship between I_{Ami} and V_{Hold}, whereas the amiloride-sensitive component of the total membrane conductance (G_{Ami}) was derived by analysis of the relationship between I_{Ami} and V_{Hold}. The effects of putative blockers of epithelial Na^+ current were quantified by analyzing their effects on the current flowing at -82 ± 5 mV (I_{-82 mV}). This potential was chosen since it equates to E_K, which implies that this current can only be carried by Na^+ and/or Cl^- . I_{-82 mV} was first measured under control conditions (i.e., [Na^+]_o = 144.4 mM), and the measurement repeated was after [Na^+]_o had been lowered to 10 mM (NMDG^+ substitution). Since this reduction in [Na^+]_o was imposed with no change in [Cl^-]_o, the [Na^+]_o-dependent component of I_{-82 mV} (I_{Na, n=10}) gives an estimate of membrane Na^+ current. All data are presented as means ± SE, and values of n refer to the number of cells in each group. All reported phenomena were observed in cells from at least three independent passages. The statistical significance of differences between mean values was assessed using Student’s unpaired t-test.

Transient transfection protocol. Cells plated onto glass coverslips and maintained in hormone-free medium (see above) for 4–6 h were transfected (Lipofectamine transfection reagent, Invitrogen) with pGL3 plasmids (Invitrogen) incorporating the appropriate cDNA constructs (1 μg; see below) in conjunction with a second plasmid (pEGFP, 0.1 μg) encoding green fluorescent protein (GFP). After 24–48 h of culture in the conditions detailed in the text, coverslips bearing these cells were mounted into a perfusion chamber attached to the stage of a Nikon inverted microscope equipped with epifluorescence optics. Membrane currents were then recorded from successfully transfected cells, which were identified by GFP fluorescence. The success of this method is based on the assumption that the GFP-expressing cells will also express the test constructs. We believe this assumption is justified as the transfection reagent used is designed to form micelle-like structures containing many plasmid DNA molecules. There is no reason why a particular type of plasmid would be excluded from these complexes, and, since the entire micelle is taken up by the transfected cells, there is no mechanism by which a cell could selectively express a GFP-encoding plasmid.

cDNA constructs. Cellular serum- and glucocorticoid-regulated kinase 1 (SGK1; see Ref. 31) activity was artificially increased by transfecting cells with a cDNA construct encoding a glutathione S-transferase (GST)-fusion protein incorporating a truncated form of SGK1 lacking 60 NH2 terminus amino acid residues that had been further modified by mutating Ser^112 to Asp (SGK1-S422D). The NH2 terminal truncation induces a 20- to 250-fold increase in protein expression by preventing degradation of the protein, whereas the S422D mutation allows this truncated protein to be more readily activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) (29, 31). Taken together, these mutations confer a constitutively active phenotype on this mutant (29). Nonspecific effects of the transfection procedure and/or expression of a heterologous protein were controlled for using a construct encoding an analogous GST-truncated SGK1 protein in which Lys^127 had been mutated to Ala (SGK1-K127A). This mutation disrupts the protein ATP-binding site, creating a catalytically inactive form of the enzyme (29). The role of phosphatidylinositol-3-kinase (PI3K) was explored using constructs encoding chimeric proteins consisting of the catalytic, P110alpha domain of PI3K (PI3K-P110alpha) attached to the extracellular and transmembrane domains of the rat CD2 surface antigen, which effectively anchors the PI3K-P110alpha subunit to the inner surface of the plasma membrane. The construct used to enhance membrane PI3K activity (rCD2-P110alpha) contained wild-type PI3K (PI3K-P110alpha) attached to the extracellular and transmembrane domains of the rat CD2 surface antigen, which effectively anchors the PI3K-P110alpha subunit to the inner surface of the plasma membrane. The construct used to enhance membrane PI3K activity (rCD2-P110alpha) contained wild-type PI3K (PI3K-P110alpha) attached to the extracellular and transmembrane domains of the rat CD2 surface antigen, which effectively anchors the PI3K-P110alpha subunit to the inner surface of the plasma membrane. The construct used to enhance membrane PI3K activity (rCD2-P110alpha) contained wild-type PI3K (PI3K-P110alpha) attached to the extracellular and transmembrane domains of the rat CD2 surface antigen, which effectively anchors the PI3K-P110alpha subunit to the inner surface of the plasma membrane.

RESULTS

Verification of transient transfection procedure. In an initial series of experiments, membrane currents were recorded from cells that had simply been transfected with GFP-expressing plasmids (see METHODS) to assess the efficacy of the transfection procedure and to determine whether exposure to the transfection reagent and/or expression of a heterologous protein had any effect on the electrical properties of the cells. These experiments showed that 20–40% of the cells expressed
sufficient GFP to be visible under epifluorescence. We often saw groups of 3–5 cells that included only a single transfected cell, and, since earlier work had indicated that such cells are electrically interconnected (14), we decided to record currents from single cells to be sure that data were derived from transfected cells. Experiments in which membrane currents were recorded from such GFP-expressing cells (identified by epifluorescence) showed that heterologous expression of this marker protein did not affect the electrical properties of hormone-deprived cells.

Properties of hormone-deprived cells. Figure 1A shows currents recorded from hormone-deprived cells that were initially bathed with the standard, Na$^{+}$- and Cl$^{-}$-rich bath solution; these data are derived entirely from single cells to facilitate comparison with data derived from transfected cells (see above). $V_m$, under these conditions, was approximately −80 mV (Fig. 1B), and this potential did not differ significantly from $E_K$ (−81.9 mV). Lowering [Na$^{+}$]o (10 mM, NMDG$^+$ substitution; Fig. 1A) or [Cl$^{-}$]o (26.5 mM, gluconate substitution) had no effect on $I_m$ and thus caused no change in $V_m$ (Fig. 1B), whereas raising [K$^+$]o to 134.5 mM (Na$^{+}$ substitution) caused depolarization (Fig. 1B). $G_K$ is therefore the dominant ionic conductance under these conditions.

Effects of insulin. Analysis of currents recorded from insulin-treated (Fig. 2A) cells showed that $V_m$ was normally approximately −60 mV; this potential differed from that measured under hormone-free conditions (see above; $P < 0.05$) and from $E_K$ ($P < 0.05$), indicating that insulin depolarizes $V_m$ by ~20 mV. Replacing [Na$^{+}$]$_o$ with NMDG$^+$ had no effect on the $I_m$ in these cells (Fig. 2A) and so caused no change in $V_m$ (Fig. 2C), whereas lowering [Cl$^{-}$]o to 26.5 mM (gluconate substitution) led to a rightward shift in the $I_m$-$V_{\text{Hold}}$ relationship (Fig. 2B). Further analysis showed that this reduction in [Cl$^{-}$]o depolarized $V_m$ by 16.9 ± 2.9 mV (Fig. 2B).

CFTR-172 (10 μM, 2–3 min), a relatively selective blocker of the Cl$^{-}$ channels associated with expression of the CFTR protein (36), had no effect ($n = 3$) on $I_{\text{Rm}}$ (control: 1.52 ± 0.47 nS per cell; CFTR-172: 1.88 ± 0.5 nS per cell) or $V_m$ (control: −62.7 ± 9.0 mV; CFTR-172: −53.0 ± 9.8 mV) in insulin-treated cells. Subsequent experiments therefore explored the effects of DPC, a nonspecific blocker of epithelial Cl$^{-}$ channels (16). The first such experiments confirmed that $V_m$ in single, hormone-deprived cells was normally approxi-
mately −80 mV, and DPC (1 mM) had no effect on $I_m$ in such cells (Fig. 3A). Studies of insulin-treated cells (Fig. 3B) confirmed the depolarizing action of this hormone ($P < 0.05$) and showed that DPC reduced $G_{Ttot}$ (control: $1.71 \pm 0.40 \text{nS per cell}$; DPC: $1.08 \pm 0.3 \text{nS per cell}; P < 0.01$) and hyperpolarized $V_m$ to a potential that was essentially identical to $E_K$ (control: $−61.1 \pm 4.3 \text{mV};$ DPC: $−86.1 \pm 4.3 \text{mV}; P < 0.02$). DPC thus reverses the insulin-induced depolarization. Analysis of $I_{DPC}$ (see METHODS) showed that this current displayed slight inward rectification and reversed at a potential essentially identical to $E_{Cl} (−39.8 \pm 2.3 \text{mV};$ Fig. 3C), an observation that confirms that $I_{DPC}$ is $Cl^-$ selective. Further analysis of the $I_{DPC}−V_{Hold}$ relationship revealed a slope conductance of $0.64 \pm 0.13 \text{nS per cell}$, and, since the corresponding value of $G_{Ttot}$ was $1.6 \pm 0.4 \text{nS per cell}$, the insulin-induced $G_{Cl}$ accounts for $\sim 40\%$ of $G_{Ttot}$ at physiologically relevant potentials.

Expression of constructs encoding wild-type and mutant PI3K-P110α. Figure 3D shows currents recorded from hormone-deprived cells expressing rCD2-P110α-R1130P, which incorporates a catalytically inactive PI3K-P110α subunit (see METHODS). These currents were essentially identical to control (Fig. 3A), and DPC (1 mM, 1–2 min) had no effect (Fig. 3D) on either $G_{Ttot}$ (control: $0.92 \pm 0.37 \text{nS per cell}$; DPC: $0.94 \pm 0.40 \text{nS per cell}$) or $V_m$ (control: $−84.9 \pm 1.6 \text{mV};$ DPC: $−79.6 \pm 3.2 \text{mV}$). Expression of this control construct therefore has no effect on the conductive properties of hormone-deprived cells. Expression of rCD2-P110α, which incorporates wild-type PI3K-P110α (47), depolarized $V_m (P < 0.02)$ to approximately $−60 \text{mV}$ (Fig. 3E) and caused a rise in $G_{Ttot}$ when compared with data from rCD2-P110α-R1130P-expressing cells ($P < 0.05$). Application of DPC inhibited $G_{Ttot}$ (control: $2.3 \pm 0.66 \text{nS per cell};$ DPC: $1.1 \pm 0.33 \text{nS per cell}; P < 0.05$) and hyperpolarized $V_m$ to a value close to $E_K$ (control: $−64.0 \pm 3.7 \text{mV};$ DPC: $−85.1 \pm 3.1 \text{mV}; P < 0.001$). Further analysis showed that expression of rCD2-P110α induced a DPC-sensitive current that was two to three-fold larger than that seen in insulin-stimulated cells but otherwise essentially identical (Fig. 3F).

**Properties of the insulin-induced Cl$^-$ conductance.** Lowering $[Cl^-]o$, to 26.5 mM by isoosmotically substituting $I^-$ hyperpolarized insulin-treated cells by $−12 \text{mV}$ (control: $−62.6 \pm 5.3 \text{mV};$ $I^−: −75.9 \pm 3.7 \text{mV}; n = 4; P < 0.05$). Although this result suggests that the insulin-induced $G_{Cl}$ may be more permeable to $I^−$ than to $Cl^−$ (see also Ref. 56), it is also possible that $I^−$ may block $G_{Cl}$, which would allow $V_m$ to move toward $E_K$. We therefore undertook further studies of insulin-treated cells that were initially bathed with $K^+$- and $Cl^−$-rich solution containing bupivacaine (3 mM), a local anesthetic that causes essentially complete block of the channels that underlie $G_K$ in resting H441 cells (24). The currents recorded under these conditions normally (i.e., when $[Cl^-]o$ was 151.5 mM) reversed at a potential close to $E_{Cl} (−39.7 \pm 2.1 \text{mV})$, and lowering $[Cl^-]o$ to 26.5 mM by isoosmotically substituting a nominally impermeant anion (gluconate) re-

![Fig. 3. N-phenylanthranilic acid (DPC)-sensitive membrane currents in single cells.](http://ajplung.physiology.org/ Downloaded from)
duced the outward current flowing at positive values of $V_{\text{Hold}}$ and depolarized $V_{\text{Rev}}$ ($P < 0.05$) to $-17.0 \pm 5.0$ mV (Fig. 4). These findings (Fig. 4) show that the current recorded under these conditions is primarily carried by anions. Replacing $\text{Cl}^-$ with $I^-$ ($\text{Cl}^-$o = 26.5 mM) enhanced the outward currents flowing at positive values of $V_{\text{Hold}}$ and hyperpolarized $V_{\text{Rev}}$ to $-51.3 \pm 3.5$ mV ($P < 0.02$; Fig. 4). Since $E_K$ was held at $\approx 0$ mV throughout all solution changes (i.e., $[K^+]_o$ was always elevated), this effect shows that exposure to the $I^-$-rich saline must augment a hyperpolarizing anion current. The channels that underlie $G_{\text{Cl}}$ must therefore be more permeable to $I^-$ than to $\text{Cl}^-$ (see also Ref. 56).

Effects of dexamethasone. Analysis of data recorded from dexamethasone-stimulated cells (Fig. 5A) showed that this synthetic glucocorticoid depolarized ($P < 0.05$) $V_m$ to a value (approximately $-60$ mV) that differed significantly ($P < 0.05$) from $E_K$. Lowering $[\text{Cl}^-]_o$ (gluconate substitution) had no effect on $V_m$ in dexamethasone-stimulated cells (Fig. 5B), whereas reducing $[\text{Na}^+]_o$ inhibited the inward currents at negative values of $V_{\text{Hold}}$ (Fig. 5A) and hyperpolarized $V_m$ (Fig. 5B). The dexamethasone-induced depolarization thus reflects a rise in $G_{\text{Na}}$ (see also Ref. 14) with no change in $G_{\text{Cl}}$. Figure 6A shows values of $I_{\text{Na}}$ derived from these data (see METHODS) and plotted against $V_{\text{Hold}}$ and also includes equivalent data from hormone-deprived cells (Fig. 1A). It is abundantly clear that $I_{\text{Na}}$ is negligible in hormone-deprived cells and that dexamethasone induces a clear current. Regression analysis indicated that this synthetic glucocorticoid increased $G_{\text{Na}}$ to $\sim 0.6$ nS per cell (Fig. 6C). Figure 6B shows equivalent data from cells stimulated with insulin either alone or in combination with dexamethasone. These data confirm that insulin does not directly induce $I_{\text{Na}}$ but establish that this hormone can augment the dexamethasone-induced $G_{\text{Na}}$ (Fig. 6, B and C).

$I_{\text{Na}}$ in cells expressing mutant forms of SGK1. Figure 6D shows the relationships between $I_{\text{Na}}$ and $V_{\text{Hold}}$ for hormone-deprived cells expressing SGK1-K127A or SGK1-S422D. $I_{\text{Na}}$ is negligible in the SGK1-K127A-expressing cells, confirming that the transfection procedure has no effect on membrane conductance. Expression of SGK1-S422D, on the other hand, induced an inward current similar to that seen in dexamethasone-stimulated cells (Fig. 6, D and F). Figure 6E shows analogous data derived from insulin-stimulated cells. Once again, SGK1-K127A expression did not induce any discernible $I_{\text{Na}}$, whereas SGK1-S422D expression evoked a substantial current (Fig. 6E). Although insulin did not induce $I_{\text{Na}}$ in SGK1-K127A-expressing cells (Fig. 6, B and C), this hormone augmented the current evoked by SGK1-S422D (Fig. 6, E and F).

$I_{\text{Na}}$ in cells expressing rCD2-P110α and rCD2-P110α-R1130P. Figure 7A shows data from rCD2-P110α-R1130P-expressing cells that had been maintained ($\sim 24$ h) in hormone-free or dexamethasone-supplemented medium. $I_{\text{Na}}$ was negligible under hormone-free conditions, and dexamethasone induced a current very similar to that seen in untransfected cells (Fig. 6A). Expression of rCD2-P110α-R1130P therefore has no effect on the conductive properties of unstimulated or dexamethasone-stimulated cells. Figure 7B shows equivalent data from rCD2-P110α-expressing cells. Although the expression of this construct, which incorporates wild-type P13K-P110α (47), did not induce $I_{\text{Na}}$ in unstimulated cells, it augmented the dexamethasone-induced $I_{\text{Na}}$ (Fig. 7C).

Properties of the hormonally induced $G_{\text{Na}}$. Figure 8A shows the effects of amiloride (10 μM) on the current recorded from single hormone-treated (0.2 μM dexamethasone, 20 nM insulin, $\sim 24$ h) cells. The mean value of $G_{\text{m}}$ associated with these recordings was thus $\sim 30$ pF. Amiloride has no effect on the

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**Fig. 4.** Bupivacaine-resistant membrane currents in single, insulin-treated cells. All solutions used in these experiments contained bupivacaine (3 mM), which was included to block $G_K$ (24). The figure shows relationships between bupivacaine-resistant component of $I_m$ and $V_{\text{Hold}}$. In each experiment, data were initially derived from cells bathed with the K+ (134.5 mM)- and Cl- (151.5 mM)-rich bath solution, which was designed to hold $E_K$ at $\approx 0$ mV, and the measurements were then repeated after [Cl-]o had been lowered to 26.5 mM by isoosmotically replacing this anion with gluconate (Gluc.) or $I^-$. All data are means ± SE.

**Fig. 5.** Electrophysiological properties of single, dexamethasone-stimulated cells. A: plot showing the relationship between $I_m$ and $V_{\text{Rev}}$ for cells ($n = 9$) maintained in dexamethasone-containing (0.2 μM) medium for $\sim 24$ h; currents were recorded under control conditions and after [Na+]o had been lowered to 10 mM (NMDG+ substitution). B: values of $V_m$ estimated from data recorded under conditions (open columns) and then after the ionic composition of the bath solution had been modified (experimental; filled columns) by lowering [Na+]o to 10 mM (low Na+; further analysis of data in A) or lowering [Cl-]o to 26.5 mM (low Cl-, $n = 4$). All data are means ± SE. ***$P < 0.01$, statistically significant difference from control.
The data in Fig. 9A were recorded from hormone-treated cells at identical passage to those that provided the data in Fig. 8, and the only difference between these experiments is that all data in Fig. 9 are derived from cells growing in groups that appeared to contain 3–5 cells. The value of $V_m$ measured in different cells (range: −20.7 to 20.2 mV), and analysis of the data from all experiments included in the present study in which membrane currents were recorded from such groups of cells showed that the mean value of $V_m$ was approximately −20 mV. This is very similar to the previously reported value (14). Under these conditions, amiloride (10 μM, 30–45 s) hyperpolarized $V_m$ to −56.7 ± 1.5 mV ($P < 0.005$) and reduced the inward currents flowing at negative values of $V_{Hold}$ (see METHODS; **$P < 0.01$, statistically significant difference from control) and $G_{Na}$ derived from analysis of the data in B and E; *$P < 0.05$, ***$P < 0.01$, statistically significant differences between SGK1-K127A and SGK1-S422D; †statistically significant effects of insulin. All data are means ± SE.
a potential close to \( E_Na \). These data thus confirm that glucocorticoids induce a selective \( G_Na \) in such cells and show that insulin does not influence the ionic selectivity of the underlying conductance. However, it was also clear that insulin could augment this dexamethasone-induced conductance (Fig. 9D).

Pharmacological properties of the hormonally induced conductance in single cells. Further studies confirmed that 1 mM amiloride had no effect on the currents recorded from single, hormone-treated (\(~24\) h, 0.2 \( \mu M \) dexamethasone, 20 \( \mu M \) insulin) cells (Fig. 10A; control \( V_m: -29.0 \pm 9.2 \) mV; amiloride \( V_m: -29.4 \pm 5.4 \) mV); further analysis of these data therefore confirmed that \( I_{Amil} \) was negligible (Fig. 10B) and revealed no significant inhibition of \( I_{Nat}(-82 \) mV) (Fig. 10C). However, subsequent application of La\(^{3+} \) (1 mM), in the continued presence of amiloride, rapidly (2–5 s) inhibited the inward current flowing at negative values of \( V_{Hold} \) and hyperpolarized (\( P < 0.05 \) \( V_m \) to \(-45.0 \pm 8.4 \) mV (Fig. 10A), indicating that this cation blocks depolarizing \( Na^+ \) entry. However, lowering \([Na^+]_o\), to 10 mM (NMDG\(^+\) substitution), in the continued presence of La\(^{3+} \), caused further inhibition of the inward current (Fig. 10A) and additional hyperpolarization (\(-72.6 \pm 7.2 \) mV; \( P < 0.05 \)), indicating that this block is incomplete. Further analysis showed that La\(^{3+} \) caused \(~70\%\) inhibition of \( I_{Nat}(-82 \) mV) (Fig. 10C), although there was some variability between cells since essentially complete block was observed in two instances. This protocol was also used to explore the effects of 15 \( \mu M \) dichlorobenzamil (DCB) and 100 \( \mu M \) pimozide, both of which have been reported to block \( Na^+\)-permeable channels in other cell types. In each such experiment, the cells were initially exposed to 1 mM amiloride, and these data consistently confirmed that this ENaC blocker had no effect on \( I_m \). However, DCB caused \(~15\%\) inhibition of
ion, suggesting that the La³⁺-induced inhibition of outward current (Fig. 10A) might be due, at least in part, to block of the selective K⁺ current, La³⁺-selective conductance. This situation was further analyzed by assigning the K⁺ permeability (P_K) of the underlying conductance a value of unity and reiteratively adjusting the Na⁺ permeability (P_Na) to identify a solution to the Goldman-Hodgkin-Katz (GHK) Equation consistent with the observed value of V_Rev. This analysis indicated that P_Na/P_K was 0.50. However, we were concerned that the La³⁺-induced inhibition of outward current (Fig. 10A) might be due, at least in part, to block of the selective K⁺

**Fig. 9.** Properties of the hormonally induced (0.2 μM dexamethasone, 20 nM insulin, −24 h) G_Na in single cells. A: in each experiment, I_m was initially recorded from groups of 3–5 cells (C_m = 71.6 ± 6.3 pF) that had been incubated (~24 h) in medium containing dexamethasone (0.2 μM) and insulin (20 nM). In each experiment, I_m was initially recorded from cells bathed with standard conditions (control), and the measurements were repeated during exposure to 10 μM amiloride and the NMDG⁺-rich, low Na⁺ solution containing 10 μM amiloride (low Na⁺). B: these data were further analyzed to isolate I_Amil and I_nNa, which are plotted (means ± SE) against V_Hold. C: plots showing the relationship between I_Amil and V_Hold derived for cells growing as small groups that had been exposed to 0.2 μM dexamethasone (~24 h) either alone (n = 6, C_m = 57.7 ± 3.1 pF) or in combination with 20 nM insulin (n = 10, C_m = 74.1 ± 5.6 pF). D: values of total membrane conductance (G_m) derived from further analysis of these data (see METHODS) and presented as means ± SE. *P < 0.05, statistically significant differences between the data derived from the 2 groups of cells.

**Fig. 10.** Properties of the hormonally induced (0.2 μM dexamethasone, 20 nM insulin, −24 h) G_Na in cells growing as small groups. A: plots showing the relationship between I_m and V_Hold derived from experiments (n = 5) in which I_m was recorded from groups of 3–5 cells (C_m = 71.6 ± 6.3 pF) that had been incubated (~24 h) in medium containing dexamethasone (0.2 μM) and insulin (20 nM). In each experiment, I_m was initially recorded under standard conditions (control), and the measurements were repeated during exposure to 10 μM amiloride and the NMDG⁺-rich, low Na⁺ solution containing 10 μM amiloride (low Na⁺). B: these data were further analyzed to isolate I_Amil and I_nNa, which are plotted (means ± SE) against V_Hold. C: plots showing the relationship between I_Amil and V_Hold derived for cells growing as small groups that had been exposed to 0.2 μM dexamethasone (~24 h) either alone (n = 6, C_m = 57.7 ± 3.1 pF) or in combination with 20 nM insulin (n = 10, C_m = 74.1 ± 5.6 pF). D: values of total membrane conductance (G_m) derived from further analysis of these data (see METHODS) and presented as means ± SE. *P < 0.05, statistically significant differences between the data derived from the 2 groups of cells.

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Ion selectivity of the La⁳⁺-sensitive conductance. As well as blocking inward Na⁺ current, La⁢³⁺ reduced the outward current flowing at positive values of V_Hold (Fig. 10A), and analysis of the I_La-V_Hold relationship (Fig. 10C) indicated that this current reversed at −10.8 ± 6.6 mV. This potential differed from E_Na, suggesting that I_La is not Na⁺-selective.
conductance ($G_K$) in these cells (24) and therefore undertook further experiments in which $I_L$ was quantified in the presence of bupivacaine (3 mM), a substance that causes essentially complete block of $G_K$ (24). This substance had no effect on $I_L$ (Fig. 10D), and the value of $V_{rev}$ derived from these experiments ($-3.8 \pm 4.4$ mV) did not differ significantly from that reported above. Analysis of these data indicated that $P_{Na/D}$ was 0.69. Moreover, raising $[K^{+}]_o$ to 134.5 mM (Na$^+$ substitution) caused a rightward shift in the $I_L$ vs. $V_{hold}$ relationship (Fig. 10D) so that $V_{rev}$ was now $6.7 \pm 3.8$ mV, and this potential did not differ significantly from that predicted by the GHK Equation (4.1 mV) for a conductance with this degree of Na$^+$ selectivity.

**Characteristics of the SGK1-S422D induced $G_{Na}$.** Further studies ($n = 5$) of SGK1-S422D-expressing cells maintained ($\sim 24$ h) in insulin-containing (20 nM) medium showed that amiloride (10 $\mu$M) had no effect on conductive properties of the plasma membrane (control $V_m = -22.7 \pm 4.5$ mV; amiloride $V_m = -21.6 \pm 3.5$ mV), whereas reducing $[Na^{+}]_o$ to 10 mM (NMDG$^+$ substitution) consistently hyperpolarized $V_m$ to $-42.7 \pm 3.9$ mV ($P < 0.05$). Amiloride also had no effect at 1 mM ($n = 5$). These studies therefore confirm that heterologous expression of SGK1-S422D induces $G_{Na}$ and show that this conductance is amiloride resistant. Further experiments showed that this SGK1-induced conductance was blocked by La$^{3+}$ ($\sim 95\%$ inhibition), pimozide ($\sim 50\%$ inhibition), and DCB ($\sim 15\%$ inhibition) (Fig. 11A), and, although this pattern is broadly similar to that seen in hormonally stimulated cells, La$^{3+}$ acted as a slightly more effective blocker of $G_{Na}$ in the SGK1-expressing cells ($P < 0.005$), causing essentially complete block in most experiments. Experiments in which $I_L$ was quantified during exposure to bupivacaine (3 mM) showed that this current normally reversed at $-12.8 \pm 1.7$ mV, and, although slightly more negative than the equivalent data derived from hormone-treated cells (Fig. 10D), this difference was not statistically significant, and analysis using the GHK Equation (see above) showed that $P_{Na/D}$ was 0.46. Exposing these cells to the K$^+$-rich bath solution depolarized $V_{rev}$ to $4.8 \pm 4.5$ mV (Fig. 11B), and this potential is essentially identical to that predicted by the GHK Equation (4.2 mV) for a conductance displaying this degree of Na$^+$ selectivity.

**DISCUSSION**

The insulin-induced depolarization. Studies of hormone-deprived cells showed that $V_m$ was essentially identical to $E_K$ and demonstrated that this potential was unaffected by large reductions in $[Na^{+}]_o$ or $[Cl^-]_o$, whereas increasing $[K^+]_o$ caused depolarization. Taken together, these studies show that $G_{Na}$ and $G_{Cl}$ are both negligible under these conditions and demonstrate that the electrical properties of hormone-deprived cells are dominated by $G_K$. However, the present estimate of $V_m$ was more negative than the value reported in a previous study in which H441 cells were maintained in glucocorticoid-free medium (14), and it is therefore interesting that insulin was routinely added to all culture media used in this earlier study whereas this hormone was omitted from the media used here (see METHODS). This raised the possibility that insulin might depolarize the cells, and this hypothesis was confirmed by experiments that showed $\sim 24$ h exposure to 20 nM insulin depolarized $V_m$ by $\sim 20$ mV. However, although insulin has been shown to stimulate Na$^+$ absorption in pulmonary (19) and renal (see, for example, Ref. 7) epithelia, lowering $[Na^{+}]_o$ did not hyperpolarize insulin-stimulated cells, whereas replacing $[Cl^-]_o$ with gluconate caused further depolarization. The insulin-induced depolarization therefore cannot be attributed to an increase in $G_{Na}$ and seems to reflect a rise in $G_{Cl}$ that would allow $V_m$ to move away from $E_K$. Subsequent studies confirmed this hypothesis by identifying an insulin-induced, DPC-sensitive current that reversed at a potential close to $E_{Cl}$ and accounted for $\sim 40\%$ of $G_{Tot}$ at physiologically relevant values of $V_{hold}$.

As far we are aware, these data provide the first description of insulin-induced $G_{Cl}$ in absorptive epithelia, although this hormone has been shown to increase $G_{Cl}$ in a liver-derived cell line, apparently by activating PI3K (28), an enzyme that catalyzes the phosphorylation of phosphatidylinositol 3-phosphate and phosphatidylinositol 3,4-biphosphate to phosphatidylinositol 4,5-biphosphate ($PIP_2$) and phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$), respectively (see, for example, Ref. 15). These anionic phospholipids control many aspects of cell physiology by activating PI3K and downstream kinases such as SGK1 (15, 29) and by directly controlling ion channel activity (see, for example, Refs. 20, 22). Since PI3K has also been reported to control $G_{Na}$ in arterial smooth muscle (13), subsequent experiments explored the effects of artificially increasing cellular PI3K activity by transiently expressing membrane-anchored PI3K-P110et (see Ref. 47). Initial studies showed that
mock transfection (i.e., heterologous expression of GFP) had no effect on the conductive properties of the cells and established that expression of a control construct encoding a catalytically inactive, membrane-anchored PI3K-P110α mutant was similarly ineffective. It is therefore clear that the transfection procedure itself has no direct effect on the conductive properties of H441 cells. However, expression of the construct encoding membrane-anchored wild-type PI3K-P110α depolarized the cells by inducing a DPC-sensitive current similar to that seen in insulin-stimulated cells. This occurred with no effect on GNa (see below), and so artificially augmenting PI3K activity mimics the depolarizing action of insulin.

The insulin/PI3K-induced conductance. We initially sought to characterize the insulin/PI3K-induced GCl by quantifying IDPC in the presence of different external anions, but this proved unfeasible as anionic substitution/DPC application frequently caused rupture of the seal between the recording pipette and the cell, making it essentially impossible to record Im throughout several such solution changes. We therefore adopted an alternative strategy based on the fact that bupivacaine blocks the channels that underlie Gk in H441 cells (24). Initial studies of bupivacaine-treated cells bathed with K+-rich physiological saline showed that Vm normally lay close to ECl. Moreover, lowering [Cl]o (gluconate substitution) caused depolarization, and these findings confirm that GCl is the dominant conductance under these conditions (24). The current recorded under these conditions displayed slight outward rectification, which was surprising since IDPC was inwardly rectifying. The reason for this discrepancy is unknown, but it may be relevant that DPC is a relatively weak anion channel blocker and that efficacies of blockade are influenced by Vm (see, for example, Ref. 44). These effects could give rise to disparities between IDPC and the true Cl− current. Although we cannot formally exclude the possibility that bupivacaine may directly modify the conductive properties of the anion channels that underlie GCl, we are not aware of any precedents for such behavior. However, the most important point to emerge from these studies was that the population of anion channels that underlie GCl was more permeable to I− than to Cl−. The insulin/PI3K-induced GCl therefore resembles an anion-selective conductance recently identified in rat cortical lens fiber cells (55). Although it has been suggested that H441 cells express significant amounts of CFTR (30, 33), these channels are characteristically less permeable to I− than to Cl−, and so the present data, in common with earlier work (56), suggest that CFTR does not contribute significantly to GCl under the present conditions. Further evidence of this came from the fact that CFTR-172, which blocks CFTR with a reasonable degree of selectivity (36), had no effect on the conductive properties of insulin-treated cells.

The dexamethasone-induced depolarization. Dexamethasone also depolarized H441 cells, but this response, in contrast to the response to insulin, involved a rise in GNa (see also Ref. 14) that occurred with no change in GCl. Moreover, this effect was reproduced by transient expression of constitutively active SGK1 (SGK1-S422D), whereas the inactive, control construct (SGK1-K127A) had no effect. These observations are consistent with a body of data that highlights SGK1 as an important regulator of GNa in absorptive epithelia (1–3, 31, 49–51, 53, 57). Although it is clear that glucocorticoid hormones do induce SGK1 expression (31), SGK1 is an important downstream target of PI3K (29), and data from several absorptive cell types have suggested that insulin can increase the apical abundance of ENaC via a mechanism dependent on a PI3K-mediated increase in SGK1 activity (2, 8, 46, 51). Moreover, PIP2 and PIP3 also activate ENaC by binding to the channel complex, and this provides another mechanism by which PI3K-coupled agonists, such as insulin, can stimulate Na+ transport (20, 42, 43, 52). However, although the present data show that insulin stimulation/PI3K-P110α expression can control GCl, these maneuvers had no effect on GNa in hormone-deprived cells but augmented the dexamethasone/SGK1-induced GNa. The present data indicate that PI3K can only control GNa in cells that have been “primed” by glucocorticoid stimulation/SGK1-S422D expression.

This was surprising since previous studies of H441 cells indicated that PI3K played a central role in the control of Na+ transport (51). However, the cells used in this earlier study displayed a Na+-absorbing phenotype under basal conditions (51), whereas, in our hands, GNa is negligible in hormone-deprived cells (see also Ref. 14). This almost certainly reflects differences in culture conditions since the earlier data (51) are derived from cells maintained in media supplemented with serum that contains a complex mixture of hormones and growth factors that can activate SGK1 (31). Serum stimulation might therefore “prime” H441 cells and thus allow PI3K to contribute control GNa. However, some renal cell lines clearly display basal Na+ transport when deprived of serum/hormones, and it is clear that insulin/PI3K can regulate Na+ transport in these cells (2, 7, 8, 37, 40), indicating that the priming effect described here is not necessary in all experimental systems. Moreover, in renal cells, insulin has no effect on the Na+ current induced by constitutively active SGK1 (2, 4), whereas the SGK1-S422D-induced Na+ current reported here was clearly enhanced by insulin. The physiological significance of these discrepancies is unknown, but they could reflect differences in culture conditions or be related to tissue-related differences in basal activity of regulatory enzymes such as PI3K/SGK1. It would therefore be interesting to record membrane currents from renal cells maintained under conditions absolutely identical to those used here.

Biophysical properties of the hormonally induced conductance. Although the hormonally induced conductance in single cells was permeable to Na+, it was unaffected by high concentrations of amiloride but could be blocked by La3+ and, to a lesser extent, by pimozone and DCB. Biophysical analysis showed that the ion channels underlying this conductance discriminated between Na+ and K+ very poorly (PNa/PK = 0.6), and so the dexamethasone-induced GNa in single cells reflects the expression of a nonselective cation conductance that is subject to further upregulation by insulin. Moreover, transient expression of constitutively active SGK1 mimicked this effect of dexamethasone, and, as far as we are aware, this observation provides the first indication that this important regulatory enzyme can control the activity of such cation channels.

We were, however, extremely surprised by these findings since Na+ entry in absorptive epithelial cells, such as H441 cells, is almost invariably attributed to the Na+–selective channels associated with α-, β-, and γ-ENaC coexpression, and these are characteristically sensitive to amiloride. Indeed, previous work from this laboratory (14) has shown that glucocor-
ticosids can induce such an ENaC-like $G_{Na}$ in H441 cells. However, almost all data in this earlier report were derived from groups of two to five electrically coupled cells, whereas membrane currents were routinely recorded from single cells in the present study. The reason for this discrepancy is that the present study aimed to explore the physiological consequences of expressing the SGK1/P13K-encoding constructs, and, since the transfection efficiency was low, we almost never observed cell clumps in which every cell was transfected. Currents were therefore recorded from single cells to ensure that all data originated from unambiguously transfected cells, and the studies of hormonally stimulated cells followed the same protocol in the interests of consistency. However, this clear discrepancy with earlier work prompted us to undertake further studies of cells growing as small groups, and these experiments clearly confirmed (14) that the glucocorticoid-induced $G_{Na}$ recorded under these conditions is $Na^+$ selective and amiloride sensitive. Moreover, the new data presented here extend on these earlier findings by showing this dexamethasone-induced conductance is further upregulated by insulin, which supports the view that this hormone might play a role in the control of pulmonary $Na^+$ transport (17–19).

It is therefore clear that hormonally stimulated H441 cells can express either an amiloride-sensitive, $Na^+$-selective conductance (14) or a La$^{3+}$-sensitive, nonselective cation conductance, and it is therefore interesting that Jain and colleagues (27) also identified such conductances in alveolar type II cells. Moreover, in this study, the relative abundance of these two channel types was strongly influenced by the culture conditions. Nonselective channels were therefore the dominant conductance in cells that had been cultured on impermeable supports using standard techniques, whereas selective ENaC-like $Na^+$ channels were the main channel type found in cells maintained in steroid-supplemented media and grown to confluence on permeable supports at an air-liquid interface (27). Similarly, studies of the $Na^+$ channels in A549 alveolar cells suggested that dexamethasone stimulation caused an increase in $Na^+$ selectivity that was associated with a reduction in single channel conductance (32). These earlier studies (27, 32) therefore suggest that hormonal stimulation, the nature of the growth substrate, the composition of the culture medium, and the methods used to culture the cells can all influence the relative expression of the selective and nonselective channels. Moreover, assays of membrane protein abundance and experiments using anti-sense oligonucleotides to suppress expression of individual ENaC subunits indicated that both of these channel types were dependent on the expression of $\alpha$-ENaC, whereas the highly selective channels required the additional expression of $\beta$- and $\gamma$-ENaC (26, 27, 32).

In the present study, however, single cells invariably displayed a nonselective conductance, whereas cells that had become integrated into small groups displayed an ENaC-like $G_{Na}$ (see also Ref. 14). Moreover, irrespective of the extent of cell-cell coupling, $G_{Na}$ was negligible in hormone-deprived cells (see also Ref. 14), and so our data indicate that the $Na^+$-selective and nonselective conductances are both induced by glucocorticoid stimulation/SGK1-S422D expression and subject to further upregulation by insulin/P13K. These conductances therefore display parallel patterns of hormonal regulation, and our data, in contrast to the earlier studies (27, 32), thus suggest that hormonal stimulation has no influence over $Na^+$ selectivity or amiloride sensitivity. Indeed, since all other culture conditions were identical, the present study indicates that electrical coupling between individual cells is the only factor that determines $Na^+$ selectivity and amiloride sensitivity. This observation, when take together with the results of earlier studies (26, 27, 32), raises the possibility that contact between individual cells might be able to influence the relative abundance of the ENaC subunits in the plasma membrane, and there are a number of precedents for such independent control over the surface expression of the ENaC subunits. For example, in alveolar type II cells, the $\beta_2$-adrenoreceptor agonist terbutaline increases in the surface abundance of $\beta$- and $\gamma$-ENaC with no effect on $\alpha$-ENaC (41). Moreover, although changes in O$_2$ tension affect the surface expression of $\alpha$-, $\beta$-, and $\gamma$-ENaC, the effect on the $\alpha$-subunit is smaller than the effect on $\beta$- and $\gamma$-ENaC (41). Furthermore, infection with Mycoplasma pulmonis has been shown to suppress the amiloride-sensitive current that can be recorded from such cells, and this inhibitory response occurs with no change to the overall abundance of the $\alpha$-, $\beta$-, and $\gamma$-ENaC proteins. However, assays of surface expression showed that infection with M. pulmonis caused a fall in the surface abundance of $\gamma$-ENaC that occurred with no effect on $\alpha$-ENaC (21). Subsequent studies of H441 cells must therefore explore the effects of hormonal stimulation, SGK1/P13K expression, and cell-cell contact on the surface expression of $\alpha$-, $\beta$-, and $\gamma$-ENaC.

**Significance of present study.** There are close parallels between the data presented here and the data presented by Shlyonsky et al. (48) who recorded currents from H441 cells grown to confluence in the presence of dexamethasone. H441 cell forms “domes” under these conditions, and these structures appear as liquid absorbed from the overlying culture medium accumulates underneath the confluent cell sheet, lifting it clear of the culture substrate. Shlyonsky et al. (48) showed that the amiloride-sensitive, selective $G_{Na}$ could only be detected in cells that formed these domes, whereas cells located away from these structures expressed the La$^{3+}$-selective, nonselective cation conductance. Moreover, these authors (48) showed that the dome-forming H441 cells express tight junctions and other markers of a polarized epithelial phenotype and therefore proposed that cell-cell communication was crucial to the development of the $Na^+$-selective, amiloride-sensitive phenotype. Although both studies (the present study and Ref. 48) suggest contact between cells is vital for the formation of amiloride-sensitive, $Na^+$-selective conductance, there are differences between the two sets of experiments. Most importantly, our data are derived entirely from subconfluent cells, and so we did not record any currents from cells in the dome-like structures described by Shlyonsky et al. (48). It therefore appears that H441 cells can be more readily induced to express the highly selective $G_{Na}$ in our hands, and this may well be a consequence of small differences in culture conditions. Interestingly, studies of mammary epithelial cells indicate that the process of dome formation is dependent on increased expression of $\beta$-ENaC. Normally, the expression of this gene appears to be repressed by a mechanism dependent on the product of a second gene, called 133, which encodes a separate, transmembrane protein (58), and dome formation appears to occur when 133 expression is repressed, which allows unimpeded $\beta$-ENaC expression (58). The way in which this process relates to the effects of cell contact described in the
electrophysiological studies (the present study and Ref. 48) here is uncertain.

The present data confirm that glucocorticoids induce $G_{Na}$ in hormone-deprived H441 cells (14) and extend on these findings by showing that this effect is mimicked by heterologous expression of active SGK1 and by demonstrating that this glucocorticoid-induced conductance is subject to further regulation via insulin/PI3K. However, the present data also show that electrical coupling between cells exerts a powerful influence over the biophysical properties of this hormonally induced $G_{Na}$, and such cell-cell interaction thus appears essential for the expression of the Na$^+$-selective, amiloride-sensitive channels that permit Na$^+$ entry in absorptive tissues.

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