The phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) is accepted to be a direct modulator of ion channel activity. The products of phosphatidylinositol 3-OH kinase (PI3K), PtdIns(3,4)P$_2$ and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$), in contrast, are not. We report here activation of the epithelial Na$^+$ channel (ENaC) reconstituted in Chinese hamster ovary cells by PI3K. Insulin-like growth factor-I also activated reconstituted ENaC and increased Na$^+$ reabsorption across renal A6 epithelial cell monolayers via PI3K. Neither IGF-I nor PI3K affected the levels of ENaC in the plasma membrane. The effects of PI3K and IGF-I on ENaC activity paralleled changes in the plasma membrane levels of the PI3K product phospholipids, PtdIns(3,4)P$_2$/PtdIns(3,4,5)P$_3$, as measured by evanescent field fluorescence microscopy. Both PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ activated ENaC in excised patches. Activation of ENaC by PI3K and its phospholipid products corresponded to changes in channel open probability. We conclude that PI3K directly modulates ENaC activity via PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$. This represents a novel transduction pathway whereby growth factors, such as IGF-I, rapidly modulate target proteins independent of signaling elicited by kinases downstream of PI3K.

Membrane phospholipids influence the activity of several types of ion channels, including GIRK, IRK, K$_{ATP}$, ROMK, and KCNQ K$^+$ channels, as well as CNG and TRP non-selective cation channels (reviewed in Ref. 1). Emerging evidence suggests that ENaC$^1$ is similarly modulated by phospholipids (2, 3). Most phospholipid-modulated channels, including ENaC, respond directly to PtdIns(4,5)P$_2$ with this phospholipid countering channel run-down and thus, being permissive for normal channel function. Defects in channel-phosphoinositide interactions, importantly, have been shown to lead to disease (4).

ENaC is a non-inactivating, voltage-independent, highly Na$^+$-selective ion channel localized to the apical plasma membrane of electrically tight epithelial cells, such as that lining the distal renal nephron and colon, alveolar sacs, salivary glands, and sweat ducts (5, 6). ENaC, together with neuronal Degenerin channels, comprise the ENaC/Deg superfamily of ion channels. ENaC activity is rate-limiting for vectorial Na$^+$ reabsorption across transporting epithelia; consequently, its activity is a critical determinant of fluid movement and epithelia surface hydration (reviewed in Refs. 7 and 8). The significance of ENaC to human health is highlighted by inherited diseases caused by: 1) gain-of-function mutations in this channel (e.g. Liddle’s syndrome) and its upstream regulatory pathways, manifested by inappropriate Na$^+$ conservation and eventual hypertension and 2) loss-of-function mutations in the channel and its regulatory pathways (e.g. pseudohyopaldosteronism), manifested by hypotension and electrolyte disorders (9–11). Recent findings, moreover, suggest that improper activation of ENaC plays a pathological role in cystic fibrosis and polycystic kidney disease, as well as in several forms of acute respiratory distress (12–15).

Phosphoinositide 3-OH kinase (PI3K) is a lipid kinase that phosphorylates phosphatidylinositol 4-phosphate and PtdIns(4,5)P$_2$ at the D-3 position producing PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ (16). PI3K signaling via production of these phospholipids and subsequent activation of a downstream kinase cascade is critical for many cellular processes, including proliferation, growth, and protein synthesis. PI3K signaling, also, plays an important role in insulin, IGF-I, and aldosterone stimulated Na$^+$ reabsorption and aldosterone increases PtdIns(3,4,5)P$_3$ levels in epithelial cells (17–23). Application of PtdIns(3,4,5)P$_3$ was recently demonstrated to slow run-down of ENaC in excised patches made from renal A6 epithelial cells and to increase activity of ENaC heterologously expressed in Xenopus laevis oocytes independent of effects on channel expression levels in the plasma membrane (3). Moreover, PI3K signaling regulates several types of ion channels, often through a trophic response involving modulation of vesicular movement and/or channel synthesis (24–27). The phospholipid products of PI3K, PtdIns(3,4)P$_2$, and PtdIns(3,4,5)P$_3$ are not themselves widely considered to be direct modulators of ion channel activity.

We test here whether the phospholipid products of PI3K directly influence the activity of a membrane resident target ion channel, ENaC. In addition, we ask if IGF-I, a physiological activator of PI3K signaling known to increase Na$^+$ reabsorption (19, 21), activates ENaC through PI3K and its products, PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$. We demonstrate that ENaC is directly activated by PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ and...
that these phospholipids activate ENaC by increasing open probability. This direct effect of PtdIns(3,4)P2 and PtdIns-(3,4,5)P3 on ENaC agrees well with the previous findings of others investigating phospholipid regulation of ENaC (2, 3) and represents a novel transduction mechanism for P13K. This mechanism enables growth factors, such as IGF-I, to rapidly modulate final effectors independent of slower trophic responses elicited by downstream signaling pathways.

MATERIALS AND METHODS

Electrophysiology—The plasmids encoding human ENaC, a kind gift from P. M. Snyder, have been described previously (29). Myc-tagged ENaC constructs have been described previously (28). Cells were maintained in culture in a humidified incubator at 37 °C in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum supplemented with 10 μM amiloride. Electrophysiological measurements were made 24–72 h after transfection. Transfected cells were identified by fluorescence from co-transfected GFP. For excised inside-out patches, extracellular pipette and internal bath solutions were (in millimolar): 140 KCl, 1 MgCl2, 10 HEPES (pH 7.4) and 140 NaCl, 1 MgCl2, 2 ATP, 0.1 GTP, 5 EGTA, 10 HEPES (pH 7.4), respectively. For whole cell experiments testing IGF-I, the bath and pipette contained (in millimolar): GTP, 5 EGTA, 10 HEPES (pH 7.4), respectively. Voltage clamp experiments were performed with an EPC-9 patch clamp amplifier interfaced with a PC running PULSE (HEKA, Lambrecht, Germany) or an Axopatch 200B (Axon Instruments) interfaced with a PC running pClamp 8.1 (Axon Instruments). In some instances, a family of test pulses, stepping from a holding potential of 30 mV to −100 mV to 60 mV by increments of 20 mV, was used. For whole cell experiments, data were digitized at 2 or 5 kHz and filtered at 1 kHz. For excised patch recordings, data were digitized at 500 Hz and filtered at 100 Hz. In some instances, data were subsequently filtered with EPC-9 or EPC-8 and recorded at 1 kHz for display. In excised patch, channel activity was expressed as NP∞ − Ip∞, where Ip∞ is mean current in a patch and NP∞ is unitary current as determined by all-point histograms. Water-soluble, short-chain, dioctanoyl phosphoryl lipids were from Echelon Biosciences Inc. (Salt Lake City, UT). Aqueous phospholipid stocks prepared were filtered at 1 μm by sonication for 30 min at 4 °C and stored at −70 °C. For outside-out experiments, stock phospholipids were mixed just prior to use with an equal volume of a carrier solution containing histone H1 (0.2 mg/ml, Echelon Biosciences Inc.) and sonicated again for 10 min. Phospholipids were sonicated and applied to the bath of inside-out patches in the absence of carrier.

Renal A6 epithelial cells were cultured in complete media supplemented with aldosterone as described previously (23, 30, 31). Open circuit current across A6 cell monolayers in response to IGF-I in the absence and presence of wortmannin was quantified in a paired manner using standard methods (23, 30, 31).

Imaging Experiments—We used evanescent field microscopy to selectively illuminate the plasma membrane to quantify the membrane levels of specific phospholipids. Fusion proteins of EGFP and the pleckstrin-homology (PH) domain of phospholipid-binding proteins (e.g. Akt and PLCγ) were used as specific phospholipid reporters. Cells used for real-time evanescent field microscopy were transfected with plasmids encoding either a membrane-localized variant of EGFP (EGFP-F, Clontech) plus DrsRed2 (Clontech), or fusion proteins containing EGFP fused with either the PH domain of Akt/PKB, which senses PtdIns(3,4)P2 or PtdIns(3,4,5)P3, or of PLCγ, which senses PtdIns(4,5)P2, in the absence and presence of constitutively active PI3K and the Mγ muscarinic receptor. The Mγ muscarinic receptor was used to introduce a mechanism for decreasing PtdIns(4,5)P2 levels via activation of endogenous PLC. The cDNAs encoding PI3K fusion proteins were a kind gift from T. Meyer and have been described previously (32). Methods used in the current study followed closely those described previously by W. Almers and colleagues (33, 34). In brief, evanescent field illumination was generated with through-the-lens total internal reflection fluorescence (TIRF). Samples were viewed through a Plan Apo TIRF 60× oil-immersion, high resolution (0.15 numerical aperture) objective on a Nikon Eclipse (Nikon Instruments, Melville, NY) inverted microscope. DrsRed and EGFP were excited with green helium/neon and argon lasers, respectively, with appropriate dichroic mirrors and emission filters. This system was also interfaced with a mercury lamp with appropriate excitation and emission filter sets enabling wide-field epifluorescence imaging of DrsRed2 and eGFP-F. Images were collected and processed with a Cascade Photometric charge-coupled device camera (Bero Scientific, Tucson, AZ) interfaced with a PC running MetaMorph software.

CHO cells used for real-time indirect epifluorescence microscopy were transfected with the Akt/PKB PH domain-EGFP fusion protein plus constitutively active P13K and imaged using a 60× (1.3 numerical aperture) oil-immersion lens on a Nikon Eclipse TE2000 inverted microscope fitted with a Cascade Photometric charge-coupled device camera. EGFP was visualized with a single pass emission filter in conjunction with a single pass excitation filter.

Biochemistry—All Western blot analysis and membrane labeling experiments followed established protocols (28). In brief, CHO cells overexpressing Myc-tagged ENaC subunits were extracted with detergent lysis buffer in whole cell lysates separated by SDS-PAGE. For labeling experiments, membrane ENaC was labeled with 1 μM sulfo-NHS-LC-biotin (in phosphate-buffered saline, pH 8.0) for 30 min at 4 °C and separated from whole cell lysate by streptavidin-agarose. All lanes within a gel were loaded with equal amounts of total protein. Nitrocellulose blots were probed with mouse monoclonal anti-Myc antibody and horseradish peroxidase-conjugated goat anti-mouse secondary antibody.

Statistics—All data are mean ± S.E. A p value of <0.05 was considered significant with population data, and paired data were analyzed with appropriate t tests.

RESULTS

IGF-I and PI3K Increase ENaC Activity—ENaC is a hetero-meric channel composed of three distinct but related subunits (α, β, and γ) that is blocked by the diuretic amiloride (5–7). We reconstituted human ENaC in Chinese hamster ovary (CHO) cells to examine its regulation by PI3K and phospholipids produced by this lipid kinase. Co-transfection of cDNA encoding each of the three human ENaC subunits into CHO cells resulted in large, amiloride-sensitive, inward Na+ currents measured in whole cell clamp that were absent in untransfected cells (Fig. 1, a and b). Inward Na+ currents were sensitive to amiloride in a reversible and dose-dependent manner (Fig. 1d, IC50 = 200 nM) consistent with that reported previously (5, 35, 36). We quantified ENaC activity as the amplitude of such amiloride-sensitive, inward Na+ currents. Co-expression of constitutively active PI3K together with ENaC increased amiloride-sensitive inward Na+ currents at −80 mV from 1.3 ± 0.15 (n = 16) to 4.2 ± 0.46 nA (n = 29) (Fig. 1, a–c). CHO cells in the absence and presence of P13K had little leak current with none of it sensitive to amiloride and co-expression of PI3K had no effect on cell capacitance (not shown). Whole cell I-V relations for ENaC both in the absence and presence of constitutively active PI3K showed marked Goldman rectification and a reversal potential greater than 40 mV as expected for a highly Na+ selective channel (Fig. 1b). Reconstituted ENaC, in addition, had the hallmark of greater Li+ compared with Na+ conductance and no K+ conductance (not shown).

Two structurally unrelated PI3K inhibitors, LY294002 and wortmannin, decreased ENaC activity in cells co-expressing constitutively active PI3K (Fig. 2, a–d). These compounds also decreased ENaC activity in cells not expressing exogenous PI3K, indicating basal activity of endogenous PI3K that acts on ENaC (not shown). The amiloride-sensitive, inward Na+ currents at −80 mV cells expressing constitutively active PI3K after sustained treatment (3–6 h) with wortmannin (0.2 μM) and LY294002 (50 μM) of 0.74 ± 0.17 (n = 4) and 0.72 ± 0.20 nA (n = 9), respectively, were significantly less than that observed in the absence of inhibitor (Fig. 2e). In contrast to LY294002, its inactive analog LY303511 (iLY, 50 μM) had no effect on ENaC currents (4.0 ± 1.0 nA; n = 5; Fig. 2e). Vehicle (Me2SO) also had no effect on ENaC activity, and none of these reagents affected cellular capacitance (not shown). LY294002 is a reversible inhibitor of PI3K that competes for the ATP-binding site of this enzyme (37). Thus, LY294002 was readily reversible with ENaC activity rapidly and significantly recovering to 2.5 ± 0.7 nA at 6 h with wortmannin.
Within 2 min following washout of inhibitor (Fig. 1, a and b). In contrast, ENaC activity failed to recover following removal of wortmannin (0.75 ± 0.17; n = 4; Fig. 1d), which is an irreversible inhibitor of the kinase (16). The rapid rate of recovery of ENaC activity following removal of LY294002 is consistent with PI3K activity, in our experimental model, overwhelming opposing lipid phosphatase activity. Under these conditions, as expected, acute (5 min) application of LY294002 (5.7 ± 1.1 before and 5.6 ± 1.2 nA after; n = 9) and wortmannin (3.2 ± 1.4 before and 3.3 ± 1.3 nA after; n = 3) had little effect on ENaC with their inhibitory actions presumably manifesting only after lipid phosphatases significantly reduced PtdIns(3,4)P2/PtdIns(3,4,5)P3 levels in the continued presence of inhibited kinase (see also Fig. 5d below). An alternative that cannot be excluded with the current data set is that the channel has a high affinity for phospholipids as Yue and colleagues (2) have suggested that ENaC does for PtdIns(4,5)P2 leading to a slow loss of activity upon blockade of PI3K. Nonetheless, the current results demonstrate that PI3K inhibitors do not directly block ENaC but affect the channel by modulating PI3K and that prolonged increases in PtdIns(3,4)P2/PtdIns(3,4,5)P3 levels in response to overexpression of constitutively active PI3K provide the cellular mechanism for sustained activation of the channel.

Because activation of PI3K is known to be downstream of stimulation of enzyme-linked growth factor receptors, we asked if stimulation of IGF-I receptors would also enhance activity of ENaC reconstituted in CHO cells. Indeed, bath application of IGF-I (100 ng/ml; 5 min) increased ENaC activity from 2.2 ± 0.50 to 3.7 ± 0.80 nA (n = 8; Fig. 3, a and c). Consistent with this action being through PI3K, enhanced current in response to IGF-I was sensitive to PI3K blockade by wortmannin with inward Na+ currents in wortmannin-treated cells being 1.2 ± 0.68 and 1.1 ± 0.62 nA, respectively, before and after IGF-I for 5 min in the continued presence of wortmannin (n = 3; Fig. 3, b and c).

Similar to its effects on ENaC reconstituted in CHO cells, IGF-I increased Na+ reabsorption across A6 epithelial cells. The summary graph in Fig. 3d shows the relative open circuit current across A6 monolayers before and 1 h after treatment with IGF-I (100 ng/ml) in the absence and presence of wortmannin (200 nM). Sodium reabsorption across these native epithelia significantly increased 2-fold in response to IGF-I in a PI3K-sensitive manner (n = 12).

Co-expression of constitutively active PI3K (Fig. 2f) and treatment with IGF-I for 30 min (Fig. 3e) did not affect ENaC expression levels. Moreover, constitutively active PI3K and LY294002 had no effect on the levels of ENaC in the plasma membrane (not shown). Thus, the results in Figs. 1–3 suggest that active PI3K enhances ENaC activity in a manner unrelated to any slower trophic effects and that IGF-I receptors can regulate ENaC activity via PI3K activation.
PI3K Activity Affects ENaC Open Probability—To determine whether signaling from PI3K to ENaC is membrane delimited or localized closely to the plasma membrane, we investigated the actions of PI3K on ENaC in excised, outside-out patches. Prolonged treatment (3–6 h) of cells co-overexpressing ENaC and constitutively active PI3K with wortmannin (Fig. 5a) and LY294002 (Fig. 4a) but not its inactive analog LY303511 (Fig. 5b) significantly decreased ENaC activity (NPo) compared with control (untreated cells expressing ENaC plus PI3K; summarized in Fig. 5c). The respective activities were 7.6/1.8 (n = 10) for control, 0.86/0.24 (n = 10) for wortmannin, 0.44/0.14 (n = 7) for LY294002, and 5.3/1.6 (n = 6) for LY303511. Similar to whole cell experiments, LY294002 (Fig. 4a), but not wortmannin (Fig. 5a), was readily reversible with channel activity quickly recovering to 3.90/1.10 (n = 7) upon washout of LY294002. Vehicle (Me2SO) had no effect on ENaC in outside-out patches and neither PI3K inhibitor acutely (5 min) affected activity (see Fig. 5d; NPo = 4.1 ± 0.6 before and 3.9 ± 0.91 after; n = 5, wortmannin and LY294002 results combined), again demonstrating that these reagents do not directly block this channel but decrease channel activity via modulation of PI3K. The time course of recovery of ENaC activity upon removal of LY294002 in excised patches was similar to that observed for channel recovery upon washout of inhibitor in whole cell experiments and parallelized increases in membrane PtdIns(3,4)P2/PtdIns(3,4,5)P3 (compare Figs. 2a and 4a; see also Fig. 7 below). Moreover, removal of LY294002 qualitatively increased channel open probability. ENaC in an untreated control cell is shown for comparison (in Fig. 4b). Together, these results strongly argue that PI3K and its phospholipid products are in relatively close proximity to the channel and are capable of enhancing channel activity rapidly.

ENaC Activity Reflects Membrane PtdIns(3,4)P2/PtdIns(3,4,5)P3 Levels—Having established that IGF-I and activation of PI3K strongly enhance ENaC activity, we next investigated their mechanism of action. Because PI3K activation results in production of PtdIns(3,4)P2 and PtdIns(3,4,5)P3, we first quantified changes in their membrane levels in response to washout of LY294002 from CHO cells overexpressing PI3K and to bath application of IGF-I to cells not transfected with PI3K. We reasoned that, if PI3K actions on ENaC were through its phospholipid products, then the time course of changes in ENaC activity should parallel changes in membrane phospholipid levels (Fig. 6). We assessed membrane levels of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 by imaging live CHO cells expressing a construct consisting of the pleckstrin-homology domain of Akt/PKB fused to EGFP with both evanescent field (Fig. 6b) and wide-field (Fig. 6d) fluorescence microscopy. The PH domain of Akt/PKB binds both PtdIns(3,4)P2 and PtdIns(3,4,5)P3 with little preference but does not interact with PtdIns(4,5)P2 (32). Evanescent field illumination was generated by total internal reflection fluorescence of a light beam directed at the interface...
between the glass coverslip and cellular plasma membrane at a glancing angle (33, 38). TIRF generates an evanescent field that declines exponentially with increasing distance from the interface between the cover glass and plasma membrane, illuminating only a small optical slice of the cell (~300 nm), including the plasma membrane. As shown in Fig. 6a, with TIRF illumination, we could reliably discriminate between the membrane-localized EGFP-F fluorophore from those in a cytosolic red-fluorescing protein (DsRed2). The membrane levels of Akt-PH significantly increased 1.8 ± 0.05 (n = 8)- and 2.2 ± 0.20 (n = 11)-fold, respectively, upon addition of IGF-I (100 ng/ml) and upon washout of LY294002 from cells pretreated with this antagonist for 3–6 h (Fig. 6c) indicating significant increases in the levels of PtdIns(3,4)P2/PtdIns(3,4,5)P3 at the plasma membrane in response to these maneuvers. In contrast but as expected, membrane Akt-PH levels (0.90 ± 0.03; n = 11) did not change upon washout of inactive LY303511. Also as expected, washout of LY294002 had no effect on PtdIns(4,5)P2.

**Fig. 3. IGF-I activates ENaC via PI3K.** Overlays of macroscopic currents elicited by voltage ramps applied to CHO cells expressing ENaC before and after addition of IGF-I (100 ng/ml; 5 min, a) and IGF-I plus wortmannin following a 4-h pretreatment with wortmannin (200 nM, b), and subsequently, amiloride (10 μM). c, summary graph comparing ENaC current density at −80 mV in cells co-expressing ENaC before and after treatment with IGF-I (100 ng/ml) in the absence and presence of wortmannin (200 nM). *p < 0.05 versus before. d, summary graph of relative (to time 0) open circuit current across A6 cell monolayers treated with vehicle (CON) and IGF-I for 1 h in the absence and presence of wortmannin. *p < 0.05 versus control. e, Western blot containing whole cell lysate from cells overexpressing myc-tagged α,β,γ-ENaC with and without IGF-I treatment (100 ng/ml; 30 min) in the absence and presence of wortmannin. Blot probed with anti-Myc antibody.

**Fig. 4. ENaC open probability reflects PI3K activity.** a, current recording of ENaC in an excised outside-out patch from a cell expressing both the channel and constitutively active PI3K pretreated with LY294002 for 4 h. LY294002 is washed near the beginning of this experiment (noted by arrow) with amiloride (10 μM) being added at the end of the experiment. The areas under the gray lines denoted I and II are shown at a faster timescale below with I showing the trace just preceding and continuing into washout of LY294002. The bottom most trace (b) is a control showing ENaC in a patch made on an untreated cell only expressing the channel. Inward Na+ current is down.
levels as measured by fluorescence of a construct consisting of the PH domain of PLC_H9254 fused to EGFP, which preferentially binds PtdIns(4,5)P₂ and not PtdIns(3,4)P₂/PtdIns(3,4,5)P₃. However, stimulation with the muscarinic agonist oxotremorine-M of co-transfected M₁ muscarinic receptors (M₁R) and subsequent activation of PLC did initiate depletion of PtdIns(4,5)P₂ but not PtdIns(3,4)P₂/PtdIns(3,4,5)P₃. These latter experiments served as controls demonstrating that the fluorescent reporters used in the current study are specific phospholipid probes. Comparable findings were observed with widefield epifluorescence microscopy with washout of LY294002 leading to marked increases in the levels of Akt-PH in membrane ruffles (Fig. 6, d and e). Both results show that IGF-I and relief of PI3K from LY294002 inhibition lead to elevation of membrane PtdIns(3,4)P₂/PtdIns(3,4,5)P₃ levels, with a time course very similar to the time course of ENaC activation (Fig. 7). These results suggested that PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ could directly influence ENaC activity.

**PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ Directly Affect ENaC Activity and Open Probability**—To test the possibility that PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ directly modulate ENaC, we next probed the effects of these phospholipids on the channel by applying them to excised outside-out (Figs. 8 and 9) and inside-out (Fig. 10) patches. Shown in Fig. 8 are current traces from outside-out patches containing ENaC from cells transfected with ENaC but not PI3K and pretreated with wortmannin for 3 h. Because these short-chain (dioctanoyl) phospholipids are membrane-permeant, particularly in the presence of carrier (39) as we used them here, their bath application in outside-out patches allowed free access to both sides of the patch membrane. ENaC activity (NPₒ) significantly increased from 1.7 ± 0.50 to 4.9 ± 1.5 (n = 10) and 0.86 ± 0.28 to 1.6 ± 0.48 (n = 8) upon addition of 10 μM PtdIns(3,4)P₂ (Fig. 8a) and PtdIns(3,4,5)P₃ (Fig. 8b), respectively, in the continued presence of wortmannin. Noteworthy in these experiments was the qualitative increase in channel open probability upon addition of either phospholipid similar to that observed upon relief of PI3K inhibition by LY294002. (Fig. 9 shows the all points histograms before and after addition of phospholipids for the current records shown in Fig. 8.)

Fig. 10a shows ensemble currents from eight inside-out patches containing ENaC from cells transfected with ENaC but not PI3K and pretreated with wortmannin for 3 h. Because these short-chain (dioctanoyl) phospholipids are membrane-permeant, particularly in the presence of carrier (39) as we used them here, their bath application in outside-out patches allowed free access to both sides of the patch membrane. ENaC activity (NPₒ) significantly increased from 1.7 ± 0.50 to 4.9 ± 1.5 (n = 10) and 0.86 ± 0.28 to 1.6 ± 0.48 (n = 8) upon addition of 10 μM PtdIns(3,4)P₂ (Fig. 8a) and PtdIns(3,4,5)P₃ (Fig. 8b), respectively, in the continued presence of wortmannin. Noteworthy in these experiments was the qualitative increase in channel open probability upon addition of either phospholipid similar to that observed upon relief of PI3K inhibition by LY294002. (Fig. 9 shows the all points histograms before and after addition of phospholipids for the current records shown in Fig. 8.)

**Fig. 5. ENaC is activated by PI3K.** a, current recording of ENaC in an excised outside-out patch from a cell expressing both the channel and active PI3K pretreated with wortmannin for 4 h before and after washout of inhibitor and subsequent application of amiloride (10 μM). The areas under the gray lines denoted I and II are shown at a faster timescale below. b, current recording of ENaC in an excised outside-out patch from a cell expressing both the channel and active PI3K pretreated with the inactive analog of LY (iLY30; LY303511) for 4 h before and after washout of iLY30 and subsequent application of amiloride (10 μM). The areas under the gray lines denoted I and II are shown at a faster timescale below. c, summary graph of ENaC activity in outside-out patches from control cells co-expressing ENaC plus constitutively active PI3K, and cells expressing ENaC plus constitutively active PI3K that were pretreated with wortmannin, LY294002, and LY303511 (iLY) before and after washout of inhibitor, *, versus control; **, versus before washout. d, current recording of ENaC in an excised outside-out patch from a cell expressing both the channel and active PI3K before and after acute treatment with wortmannin (0.2 and 2.0 μM) and subsequent application of amiloride (10 μM). The areas under the gray lines denoted I and II are shown at a faster timescale below.
the bath and KCl in the pipette) cation and anion gradients and held patches at 0 mV. Under such conditions, ENaC activity increased 3-fold upon addition of 1 μM PtdIns(3,4,5)P3 to the bath. In these experiments, significant run-down of ENaC activity was observed following patch excision (not shown) with phospholipid applied only after activity stabilized. Interestingly, run-down was observed only in inside-out but not outside-out patches, although the solutions bathing the intracellular face of the channel in both cases contained the same concentrations of ATP and GTP. These results provide strong evidence that the phospholipid products of PI3K directly influence ENaC activity. Our observations that application of phospholipids and relief of PI3K inhibition increase ENaC activity in excised patches suggest that ENaC open probability is directly regulated by membrane phospholipid levels, with phospholipids directly interacting with ENaC or a regulatory protein closely associated with the channel.

**DISCUSSION**

Hilgemann and Ball (40) and Huang and colleagues (41) first demonstrated that several classes of K+ channels are directly modulated by PtdIns(4,5)P2. Since these initial reports, many other laboratories have subsequently shown that a variety of ion channels, including ENaC (2, 3, 42–45), are directly modulated by PtdIns(4,5)P2 in both positive and negative manners. Compared with the relatively high levels of PtdIns(4,5)P2 in the plasma membrane, the PI3K phospholipid products, PtdIns(3,4)P2 and PtdIns(3,4,5)P3, are less abundant (16), making them attractive candidates to serve as direct physiological modulators of channel activity. The direct effects of these latter phospholipids on ion channels remain, for the most part, unrealized. Indeed, the odor-stimulated, Na+-gated channel in lobster olfactory receptor cells is currently the only channel reported to be stimulated by PI3K by direct activation in response to production of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (46). It has recently been demonstrated, however, that PtdIns(3,4,5)P3 reduces run-down of ENaC in excised patches made from renal A6 epithelial cells and activates the channel heterologously expressed in X. laevis oocytes independent of effects on channel membrane levels (3).

Compared with its phospholipid products, PI3K and its hormone activators are better documented as regulators of ion channel activity; however, most effects of PI3K on channel activity have been shown to be secondary to initiation of down-
stream signaling cascades. For instance, PtdIns(3,4,5)P3 mediates voltage-dependent control of cardiac K⁺ channels via actions on regulators of G protein signaling proteins (24); transforming growth factor β stimulates neuronal BKCa channels via control of channel translocation to the plasma membrane in response to PI3K-Akt/PKB signaling (25); IGF-I upregulates Kv potassium channels through control of channel synthesis in response to PI3K and its downstream effector kinases (47); and IGF-I promotes translocation of a calcium-permeable TRP channel, GRC, to the plasma membrane (27).

**Fig. 8.** PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ activate ENaC by increasing open probability. Current traces from excised outside-out patches containing ENaC from cells transfected with channel subunits and chronically treated with wortmannin (200 nM) before and after addition of PtdIns(3,4)P₂ (a) and PtdIns(3,4,5)P₃ (b) in the continued presence of wortmannin followed by treatment with amiloride (10 μM). Areas under the gray bars are shown at expanded timescales.

**Fig. 9.** PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ activate ENaC by increasing open probability. All point histograms before (a and c) and after (b and d) application of phospholipids for the current traces shown in Fig. 8.
Thus, many actions of PI3K on ion channels result from downstream signaling and/or trophic effects. Results from this study documenting direct PtdIns(3,4)P$_2$/PtdIns(3,4,5)P$_3$ actions on ENaC in excised patches demonstrate that PI3K signaling can, in addition, have more immediate actions on final effectors. The current results showing that membrane PtdIns(3,4)P$_2$/PtdIns(3,4,5)P$_3$ levels, measured with evanescent field fluorescence microscopy, change in parallel with channel activity also provide strong evidence that changes in the membrane levels of these phospholipids drive changes in ENaC activity.

Regulation of ENaC by PtdIns(4,5)P$_2$ was recently shown to be dependent on the presence of GTP and blocked by $\beta y$ subunits of trimeric G proteins suggesting a requirement of a G-like protein, possibly $G_{\alpha y z}$, for lipid regulation of the channel (2). The effects of the phospholipid products of PI3K on ENaC observed in the current study do not appear to require the presence of a trimeric G protein; however, our experiments cannot fully exclude this possibility. The current results, similar to the findings of Ma and colleagues (3), are most consistent with PtdIns(3,4)P$_2$/PtdIns(3,4,5)P$_3$ directly affecting ENaC open probability. Thus, we propose that one mechanism by which PI3K enhances ENaC activity is through production of PtdIns(3,4)P$_2$/PtdIns(3,4,5)P$_3$ with these phospholipids then directly interacting with the channel to enhance channel activity.

Putative phospholipid binding domains have been proposed in the cytosolic NH$_2$ terminus of both $\beta$- and $\gamma$-ENaC (2). These domains are rich in positively charged amino acids (Arg and Lys) and, in that respect, resemble phospholipid binding domains in other channels (41–43, 45). Other potential phospholipid regulatory domains in ENaC are the areas directly following the second transmembrane domain within both $\alpha$- and $\gamma$-ENaC. These cytosolic domains also contain several conserved positively charged residues, and deletion of this region in $\gamma$-ENaC results in decreased activity due to destabilization of channel openings and closings (28), a finding fitting well with the current results showing that PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ increase ENaC open probability. These regions in ENaC directly following the second transmembrane domain also resemble both, in charge and location, putative phospholipid binding domains in other two transmembrane containing channels, such as Kir6.2, IRK1, and GIRK4 channels (41–43).

The current results showing that phospholipid products of PI3K directly enhance ENaC activity represent a novel cellular mechanism, as depicted in Fig. 11, where PI3K and its activating hormones rapidly influence final effectors. This type of activation is direct and independent of slower trophic responses elicited by kinases downstream of PI3K.
REFERENCES

1. Hilgemann, D., Feng, S., and Nauhoogu, C. (2001) Science’s STKE http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2001/111/re19.
2. Yue, G., Malik, B., Yue, G., and Eaton, D. C. (2003) J. Biol. Chem. 278, 11965–11969
3. Ma, H. P., Saxena, S., and Warnock, D. G. (2002) J. Biol. Chem. 277, 7641–7644
4. Lopes, C., Zhang, H., Rohacs, T., Jin, T., and Logothetis, D. (2002) Neuron 34, 923–944
5. Canessa, C. M., Horisberger, J. D., and Rossier, B. C. (1993) Nature 361, 467–470
6. Verrey, F. (1995) J. Membr. Biol. 144, 93–110
7. Garty, H., and Palmer, L. G. (1997) Physiol. Rev. 77, 359–396
8. Stockand, J. D. (2002) Am. J. Physiol. 282, F559–F576
9. Hansson, J. H., Nelson-Williams, C., Suzuki, H., Schild, L., Shimkets, R., Lu, Y., Canessa, C., Iwasaki, T., Rossier, B., and Lifton, R. P. (1995) Nat. Genet. 11, 76–82
10. Schild, L., Canessa, C. M., Shimkets, R. A., Gautschi, I., Lifton, R. P., and Rossier, B. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5699–5703
11. Lifton, R. P., Gharavi, A. G., and Geller, D. S. (2001) Cell 104, 545–556
12. Geller, D. S., Rodriguez-Seriano, J., Vallo, B. A., Schiffer, S., Bayer, M., Chang, S. S., and Lifton, R. P. (1998) Nat. Genet. 19, 279–281
13. Chang, S. S., Grunder, S., Hanakogue, A., Rosler, A., Mathew, P. M., Hanakoge, I., Schild, L., Lu, Y., Shimkets, R. A., Nelson-Williams, C., Rossier, B. C., and Lifton, R. P. (1996) Nat. Genet. 12, 248–253
14. Rohatgi, R., Greenher, A., Barrow, G., Wilson, P., and Satlin, L. (2003) J. Am. Soc. Nephrol. 14, 827–836
15. Stutts, M. J., Rossier, B. C., and Boucher, R. C. (1997) J. Biol. Chem. 272, 14037–14040
16. Fruman, D., Meyers, R., and Cantley, L. (1998) Annu. Rev. Biochem. 67, 507
17. Blazer-Yost, B. L., Paunesuc, T. G., Helman, S. I., Lee, K. D., and Vlahos, C. J. (1999) Am. J. Physiol. 277, C531–C536
18. Blazer-Yost, B. L., Record, R. D., and Oberleithner, H. (1996) Pflugers Arch. 432, 685–691
19. Blazer-Yost, B. L., Esterman, M. A., and Vlahos, C. J. (2003) Am. J. Physiol. 284, C1625–C1633
20. Paunesuc, T. G., Blazer-Yost, B. L., Vlahos, C. J., and Helman, S. I. (2000) Am. J. Physiol. 279, C236–C247
21. Record, R. D., Freslich, L. L., Vlahos, C. J., and Blazer-Yost, B. L. (1998) Am. J. Physiol. 274, E611–E617
22. Wang, J., Barbry, P., Maiyari, A. C., Rozansky, D. J., Bhargava, A., Leong, M., Firestone, G. L., and Pearse, D. (2001) Am. J. Physiol. 280, F303–F313
23. Tong, Q., Booth, R. E., Worrell, R. T., and Stockand, J. D. (2004) Am. J. Physiol., in press
24. Ishii, M., Inanobe, A., and Kurachi, Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4325–4330
25. Ljubliner, L., and Dryer, S. (2002) J. Neurophysiol. 88, 954–964
26. Gamper, N., Pillon, S., Huber, S., Feng, Y., Kobayashi, T., Cohen, P., and Lang, F. (2002) Pflugers Arch. 443, 625–635
27. Kanzaki, M., Zhang, Y.-Q., Mashima, H., Li, H., Shibata, H., and Kojima, I. (1999) Nat. Cell Biol. 1, 165–170
28. Booth, R. E., Medina, J., Snyder, P. M., Patel, P., and Stockand, J. D. (2003) J. Biol. Chem. 278, 41367–41379
29. McDonald, F. J., Price, M. P., Snyder, P. M., and Welsh, M. J. (1995) Am. J. Physiol. 268, C1157–C1163
30. Booth, R. E., and Stockand, J. D. (2003) Am. J. Physiol. 284, F938–F947
31. Stockand, J. D., Spier, B. J., Worrell, R. T., Yue, G., Al-Baldawi, N., and Eaton, D. C. (1999) J. Biol. Chem. 274, 35449–35454
32. Haugh, J., Codazzi, F., Teruel, M., and Meyer, T. (2000) J. Cell Biol. 151, 1269–1280
33. Steyer, J. A., and Almers, W. (2001) Nat. Rev. Mol. Cell. Biol. 2, 268–275
34. Taraska, J. W., Perrais, D., Ohara-Imaizumi, M., Nagamatsu, S., and Almers, W. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2070–2075
35. Ishikawa, T., Marunaka, Y., and Rotin, D. (1998) J. Gen. Physiol. 111, 825–846
36. Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D., and Rossier, B. C. (1994) Nature 367, 463–467
37. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
38. Merrifield, C. J., Feldman, M. E., Wan, L., and Almers, W. (2002) Nat. Cell Biol. 4, 691–698
39. Ozaki, S., DeWald, D., Shope, J., Chen, J., and Prestwich, G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11286–11291
40. Hilgemann, D., and Ball, R. (1996) Science 273, 956–959
41. Huang, C.-L., Feng, S., and Hilgemann, D. W. (1998) Nature 391, 803–806
42. Loussouarn, G., Park, K.-H., Bellocq, C., Baro, I., Charpentier, F., and Escande, D. (2003) EMBO J. 22, 5412–5421
43. Zhang, H., He, C., Yan, X., Mirshahi, T., and Logothetis, D. (1999) Nat. Cell Biol. 1, 183–188
44. MacGregor, G., Dong, K., Vanoye, C., Tang, L., Giebisch, G., and Hebert, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2726–2731
45. Rohacs, T., Lopes, C., Jin, T., Ramdya, P., Molnar, Z., and Logothetis, D. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 745–750
46. Zhaianazarov, A., Doolin, R., Herlihy, J.-D., and Ache, B. (2001) J. Neurophysiol. 85, 2537–2544
47. Macrez, N., Mironneau, C., Carriçaburu, V., Quignard, J.-F., Babich, A., Czupalla, C., Nurnberg, B., and Mironneau, J. (2001) Circ. Res. 89, 692–699

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