Rapid Translocation and Insertion of the Epithelial Na\textsuperscript{+} Channel in Response to RhoA Signaling*

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Activity of the epithelial Na\textsuperscript{+} channel (ENaC) is limiting for Na\textsuperscript{+} absorption across many epithelia. Consequently, ENaC is a central effector impacting systemic blood volume and pressure. Two members of the Ras superfamily of small GTPases, K-Ras and RhoA, activate ENaC. K-Ras activates ENaC via a signaling pathway involving phosphatidylinositol 3-kinase and production of phosphatidylinositol 3,4,5-trisphosphate with the phospholipid directly interacting with the channel to increase open probability. How RhoA increases ENaC activity is less clear. Here we report that RhoA and K-Ras activate ENaC through independent signaling pathways and final mechanisms of action. Activation of RhoA signaling rapidly increases the membrane levels of ENaC likely by promoting channel insertion. This process dramatically increases functional ENaC current, resulting in tight spatial-temporal control of these channels. RhoA signals to ENaC via a transduction pathway, including the downstream effectors Rho kinase and phosphatidylinositol-4-phosphate 5-kinase. Phosphatidylinositol 4,5-biphosphate produced by activated phosphatidylinositol 4-phosphate 5-kinase may play a role in targeting vesicles containing ENaC to the plasma membrane.

Small G proteins act as GTP-dependent switches to control the activity of effector proteins and thus initiate cellular signaling cascades. A wide variety of ion channels and intracellular processes respond to signaling from small G proteins in the Ras superfamily (1, 2). Although distinct small G proteins can target a common effector, their actions on this common effector often vary. For example, RhoA and Rac1 have opposing actions on ether a-go-go related gene (ERG) K\textsuperscript{+} channels, with the former rapidly activating the channel and the latter quickly decreasing channel activity (3). RhoA but not closely related Rac1 and Cdc42 modulate I\textsubscript{Na} in ventricular myocytes (4). H-Ras and Rap1 function as counteracting regulators of voltage-gated sodium current and N-methyl-D-aspartic acid receptor-mediated synaptic transmission (5, 6). Similarly, Ras and Rap have opposite actions on muscarinic K\textsuperscript{+} channels (7).

We showed previously that K-Ras and RhoA increase ENaC\textsuperscript{2} activity (8, 9). Although both of these GTPases increase ENaC activity, they do so most likely through distinct mechanisms of action and signaling pathways.

The amiloride-sensitive epithelial sodium channel is localized to the luminal plasma membrane of epithelial cells and plays an important role in maintaining Na\textsuperscript{+} homeostasis and hence blood pressure (10–13). ENaC activity is dynamically controlled by regulation of channel open probability and localization to the luminal plasma membrane.

Several lines of evidence indicate that K-RasA ultimately increases ENaC single channel open probability through its first effector PI3K (9, 14). In contrast, RhoA may increase channel activity by increasing membrane levels of ENaC (8). The exact signaling pathway underlying this effect of RhoA remains to be delineated. Moreover, it is unclear whether there is signaling convergence between K-Ras and RhoA pathways with respect to control of ENaC.

One important cellular action of Rho GTPases is their regulation of actin polymerization (15). Rho proteins also participate in regulation of cell polarity and vesicle transport (16). Recently, it was shown that the Rho family GTPase Rac1 mediates rapid insertion of TRPC5 channels into the plasma membrane through stimulation of PI(4)P5K (17). We test here whether RhoA has a similar mechanism of action on ENaC.

We find that K-Ras and RhoA increase ENaC activity independent of each other through distinct signaling cascades and final mechanisms of action. RhoA increases ENaC activity through its downstream effectors Rho kinase and PI(4)P5K. RhoA signaling increases ENaC activity by elevating plasma membrane levels of the channel likely resulting from increased channel insertion.

MATERIALS AND METHODS

cDNA Constructs and Cell Culture—All chemicals and materials were purchased from either Calbiochem, Bio-Rad, Fisher,

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* This work was supported by NIDDK Grant RO1-DK-59994 from the National Institutes of Health, American Heart Association-Texas Affiliate Grant 0355012Y (to J. D. S.), and a research fellowship from the National Kidney Foundation (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be solely to indicate this fact.

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RhoA Promotes ENaC Insertion

Electrophysiology—Whole-cell macroscopic current recordings of mENaC expressed in CHO cells were made under voltage clamp conditions using standard methods (19–21). In brief, current through mENaC was the inward, amiloride-sensitive Na⁺ current at −80 mV with a bath solution (in mM) of 160 NaCl, 1 CaCl₂, 2 MgCl₂, and 10 HEPES (pH 7.4) and a pipette solution (in mM) of 120 CsCl, 5 NaCl, 2 MgCl₂, 5 EGTA, 10 HEPES, 2.0 ATP, and 0.1 GTP (pH 7.4). Where specified, cells were incubated with 1 μM Y-27632 ((+)-R-trans-4-(aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide 2HC1 H₂O), a Rho kinase (in mM) of 120 CsCl, 5 NaCl, 2 MgCl₂, 5 EGTA, 10 HEPES, 2.0

activity is reported as the amiloride-sensitive current density at −80 mV. Whole-cell capacitance was routinely compensated and was −8–10 pF for CHO cells. Series resistances, on average 2–5 megohms, were also compensated.

Trans-epithelial Na⁺ current across A6 cell monolayers was calculated, as described previously (22, 23), from Ohm’s law as the ratio of trans-epithelial voltage to trans-epithelial resistance under open circuit conditions using a Millicel electrical resistance system with dual Ag/AgCl pellet electrodes (Millipore Corp., Billerica, MA) to measure voltage and resistance. For these experiments, A6 cells were grown on Costar Transwell permeable supports (0.4 μm pore, 24 mm diameter) in the presence of 1.5 μM aldosterone and allowed to become confluent as determined by persistent and avid Na⁺ reabsorption.

Western Blot Experiments—Western blot experiments were performed using standard methods (22, 23). In brief, cells were extracted for >2 h at 4 °C in RIPA lysis buffer (10 mM NaPO₄, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS (pH 7.2)) supplemented with 1 μM of the protease inhibitor phenylmethylsulfonyl fluoride. For each lysate, protein concentration was determined with the BCA protein assay (Pierce). Following normalization of total protein concentration, and addition of sample buffer (0.005% bromphenol blue, 10% glycerol, 3% SDS, 1 mM EDTA, 77 mM Tris-HCl, and 20 mM dithiothreitol), proteins were then separated by standard SDS-PAGE (15% gels) and subsequently electrobotted to nitrocellulose (0.45-μm pore). Blots were probed with anti-HA and anti-Myc antibodies from Clontech and Roche Applied Science, respectively, in the presence of 0.1% Tween 20 and 5% dried milk as blocking reagents.

Total Internal Reflection Fluorescence Microscopy—Fluorescence emissions from eYFP-tagged ENaC (each subunit tagged with eYFP) and membrane-localized eCFP-M were collected using TIRF microscopy (30, 31) to selectively illuminate the plasma membrane. This isolated fluorescence emissions from eYFP-tagged membrane ENaC. All TIRF experiments were performed in the TIRF microscopy core facility housed within the Department of Physiology at the University of Texas Health Science Center, San Antonio. Fluorescence emissions from eYFP-tagged ENaC were collected using an inverted TE2000 microscope with through-the-lens TIRF imaging (Nikon, Japan). Samples were viewed through a plain Apo TIRF ×60 oil immersion, high resolution (1.45 N.A.) objective. The eCFP and eYFP fluorophores were excited with a 442-nm Melles Griot dual-pulsed solid state and 514-nm argon ion laser, respectively, with an acoustic optic tunable filter used to select excitation wavelengths (Prairie Technology, Middleton, WI). Emissions from eCFP and eYFP fluorophores passed through an image splitting device (Dual-View, Optical Insights, Tucson, AZ) using a 505-nm dichroic filter to split emissions, which then passed through 470 ± 15 and 550 ± 25 nm emission filters, respectively. Fluorescence images were captured with a charge-coupled device camera interfaced to a PC running Metamorph software.

Statistics—All patch clamp data are presented as mean ± S.E. Data compared with either the Student’s (two-tailed) t test or a one-way analysis of variance in conjunction with the Dunnett
K-RasG12V and RhoAG14V significantly increase ENaC activity. (Fig. 1 shows representative macroscopic ENaC currents before expressing the channel alone (Western blots for cells transfected with increasing quantities of co-expressed mENaC with RhoA and K-Ras to investigate the whether these signaling molecules were part of a converging or co-expressed mENaC and ENaC + K-RasG12V, and mENaC + RhoAG14V. C, Western blots containing lysates (100 μg of total protein) from control (Con) CHO cells and cells expressing HA-tagged K-RasG12V (1.0 μg/35-mm dish) and Myc-tagged RhoA (1.0 μg/35-mm dish) alone and together. Top and bottom blots probed with anti-HA and anti-Myc antibodies, respectively. D, dose-response curves showing the mean amiloride-sensitive current density at −80 mV for voltage-clamped CHO cells expressing mENaC in the absence and presence of K-RasG12V and RhoAG14V alone and together. The numbers of observations for each group are shown. *, significant versus mENaC alone; **, versus mENaC, mENaC + K-RasG12V, and mENaC + RhoAG14V. C, Western blots containing lysates (100 μg of total protein) from control (Con) CHO cells and cells expressing HA-tagged K-RasG12V (1.0 μg/35-mm dish) and Myc-tagged RhoA (1.0 μg/35-mm dish) alone and together. Top and bottom blots probed with anti-HA and anti-Myc antibodies, respectively. D, dose-response curves showing the mean amiloride-sensitive current density at −80 mV for voltage-clamped CHO cells co-transfected with ENaC and increasing quantities of K-RasG12V (left) and RhoAG14V (right) cDNA (for each point n = 10). E, corresponding Western blots from CHO cells transfected with increasing quantities of HA-K-RasG12V (top) and Myc-RhoAG14V (top) cDNA. Blots are representative of n = 3.

or Student Newman Keuls post-test where appropriate. p ≤ 0.05 is considered significant.

RESULTS

RhoA and K-Ras Have Independent Effects on ENaC—We co-expressed mENaC with RhoA and K-Ras to investigate the effects of these small G proteins on the channel and to ask whether these signaling molecules were part of a converging or parallel transduction pathway with respect to activating the channel. ENaC was reconstituted by co-expressing α-, β-, and γ-mENaC subunits as described previously (19–21). Fig. 1A shows representative macroscopic ENaC currents before (arrows) and after treatment with amiloride in CHO cells expressing the channel alone (top) and with constitutively active K-RasG12V (top middle) and RhoAG14V (bottom middle) separately and together (bottom). For these experiments, plasmids encoding the small G proteins were transfected at saturating levels (1.0 μg; see Fig. 1, D and E). As summarized in Fig. 1B, K-RasG12V and RhoAG14V significantly increase ENaC activity. Moreover, K-RasG12V and RhoAG14V, when expressed together, have additive effects on ENaC. The representative Western blots in Fig. 1C show that exogenous K-RasG12V (top) and RhoAG14V (bottom) were appropriately expressed in these cells. Fig. 1D shows summary graphs of ENaC activity in the presence of increasing quantities of co-transfected K-RasG12V (left) and RhoAG14V (right) cDNA. The effects of both small G proteins saturated at ~1.0 μg of cDNA. Fig. 1E shows corresponding Western blots for cells transfected with increasing quantities of K-RasG12V (top) and RhoAG14V (bottom) cDNA. These results demonstrate that at saturating levels, RhoA and K-Ras have additive and likely independent actions on ENaC consistent with these small G proteins being in parallel with respect to channel regulation.

Rho Kinase and PI(4)P5K Are Positioned between RhoA and ENaC—We next delineated the signaling pathway by which RhoA increases ENaC activity. Rho kinase is a 1st effector of RhoA (32). As shown in Fig. 2A, co-expression of constitutively active Rho kinase (Rho kinase CA) significantly increased ENaC activity. In contrast, dominant negative Rho kinase (Rho kinase DN) had little effect on basal ENaC activity. Dominant negative Rho kinase co-expressed with RhoAG14V, however, did counter activation of ENaC by this small G protein. In contrast, co-expression of dominant negative RhoA T19N with Rho kinase CA had little effect on activation of ENaC by the kinase. These results position Rho kinase downstream of RhoA in the signaling pathway from this small G protein to ENaC.

Because PI(4)P5K is an effector of RhoA and Rho kinase (33, 34), we asked whether this phospholipid kinase is involved in RhoA regulation of ENaC. Fig. 2B shows that expression of PI(4)P5K with ENaC significantly increased channel activity. Co-expression of RhoA T19N and Rho kinase DN did not influence the stimulatory effect of PI(4)P5K on ENaC. These results are consistent with PI(4)P5K being downstream of RhoA and Rho kinase in a signaling pathway leading to activation of ENaC.
RhoA and K-Ras Signaling Do Not Converge Prior to Activating ENaC—Results in Fig. 2 demonstrate that RhoA signals to ENaC via Rho kinase and PI(4)P5K. We demonstrated previously that K-Ras signals to ENaC via its 1st effector PI3K (9, 14). To further test whether RhoA and K-Ras signaling converge prior to activating ENaC, we probed the effects of co-expressing these small G proteins and their downstream effectors on ENaC activity. As summarized in Fig. 3, A, ENaC activity in the presence of PI(4)P5K plus Rho kinase CA was not different compared with mENaC plus K-RasG12V and RhoAG14V. However, ENaC activity in the presence of PI(4)P5K and Rho kinase CA was greater compared with mENaC expressed with either PI3K or Rho kinase CA alone (not shown in the same figure, see also Figs. 2 and 3B). Moreover, co-expression of PI3K with PI(4)P5K had an additive effect greater than expression of either phospholipid kinase alone (not shown). These results demonstrate that co-expression of downstream effectors of RhoA and K-Ras recapitulate independent activation of ENaC by these small G proteins. To further explore these two signaling pathways, we tested the effects of co-expression of PI(4)P5K with K-RasG12V and RhoAG14V. ENaC activity in the presence of PI(4)P5K with K-RasG12V was, as expected, significantly greater compared with mENaC expressed with either PI(4)P5K or K-RasG12V alone (see Figs. 1 and 2). In contrast, ENaC activity in the presence of both PI(4)P5K and its upstream regulator, RhoAG14V, was not different compared with expression of this channel with either alone (see Figs. 1 and 2) but was significantly lower compared with ENaC + K-RasG12V + PI(4)P5K. Similarly, we have previously demonstrated that PI3K and its upstream regulator, K-Ras, do not have additive effects on ENaC activity (9). Thus, the results in Fig. 3A are also consistent with RhoA and K-Ras signaling being independent and in parallel with respect to activation of ENaC.

As a final test of this idea, we asked if RhoAT19N disrupts ENaC activation by PI3K. Shown in Fig. 3B is a summary graph of ENaC activity in response to co-expression of constitutively active PI3K alone and with RhoAT19N. Dominant negative RhoAT19N had no effect on PI3K-stimulated ENaC.

RhoA Signaling Rapidly Increases Membrane ENaC Levels—Because RhoA and K-Ras increase ENaC activity through distinct and independent signaling pathways, we wondered if they also had different final mechanisms of action. To test whether RhoA increases ENaC activity by influencing membrane levels of the channel rather than open probability, we directly monitored changes in membrane levels of eYFP-tagged ENaC in living cells with TIRF microscopy. For these experiments, we co-expressed eYFP-tagged mENaC (all three subunits tagged) with a membrane marker (eCFP-M) in the presence and absence of RhoAG14V. Because eYFP is genetically linked to channel subunits and only membrane fluorescence is monitored with TIRF microscopy, fluorescence emissions from eYFP-ENaC are directly proportional to the number of channels in the membrane. For these experiments, cells were pretreated for 40 min with 1 μM of the Rho kinase inhibitor Y-27632 to block RhoA signaling to ENaC. Y-27632 is a competitive inhibitor of Rho kinase that is reversible (35). Changes in the levels of ENaC and the negative control eCFP-M in the membrane were monitored following washout of Y-27632 to acutely activate RhoA signaling. Fig. 4A shows representative micrographs of eYFP (top) and eCFP (bottom) fluorescence emissions from the plasma membrane of a COS-7 cell expressing eYFP-tagged ENaC and eCFP-M before (left) and after (right) acute activation of RhoA signaling upon Y-27632 washout. As summarized in Fig. 4B, the relative (compared with cells not washed of Y-27632) increase in ENaC membrane levels upon acute activation of RhoA signaling in cells expressing activated RhoA (n = 13 and 10 for washed and unwashed) was markedly greater compared with cells not expressing this small G protein (n = 7 and 9 for washed and unwashed). Relative membrane ENaC levels significantly increased 2-fold upon relief of Rho kinase inhibition in cells expressing RhoAG14V (2.0 ± 0.11 at t = 40 min). This increase is greater than the 1.2-fold increase observed in cells not expressing RhoAG14V (1.2 ± 0.10 at t = 40 min). In contrast to ENaC, acute activation of RhoA signaling had no effect on eCFP-M levels.
RhoA Promotes ENaC Insertion

Acute activation of RhoA signaling rapidly increases ENaC membrane levels. $A$, fluorescence micrographs of the plasma membrane of COS-7 cells expressing eYFP-tagged mENaC, eCFP-M, and RhoAG14V before (left) and after (right) washout of Y-27632. Cells were pretreated with 1 µM Y-27632 for 40 min. TIRF microscopy was used to focus on the plasma membrane, eYFP and eCFP emissions are shown in the top and bottom micrographs, respectively, with exposure times and gains constant before and after washout. $B$, time course of the change in relative ENaC levels in the plasma membrane of COS-7 cells expressing eYFP-ENaC in the absence (closed squares) and presence (closed circles) of RhoAG14V following washout of Y-27632. Cells were pretreated with the Rho kinase inhibitor for 40 min prior to washout, and emissions from membrane eYFP-ENaC were collected with TIRF microscopy. Membrane ENaC levels were normalized to the membrane levels of the channel in the presence of Y-27632 just prior to washout. Data points fit with an exponential rise to maximum equation. Open squares and circles show the change in relative membrane levels of eCFP-M in the absence and presence of RhoAG14V, respectively, upon washout of Y-27632. $C$, fluorescence micrographs of the plasma membrane of a COS-7 cell expressing eYFP-tagged mENaC and RhoAG14V before (left-most panel) and 6, 12, and 18 min after washout of the Rho kinase inhibitor Y-27632. This cell was pretreated with 1 µM Y-27632 for 40 min. TIRF microscopy was used to focus on the plasma membrane. Exposure time and gain was held constant over the course of these experiments. The top and bottom rows show expanded and close-up images. $D$, time course of the change in relative membrane ENaC levels following washout of Y-27632 in cells expressing eYFP-ENaC plus RhoAG14V not pretreated (open squares) and pretreated (closed squares) with 10 ng/µl brefeldin A for 22 h. Prior to washout, cells were pretreated with Y-27632 for 40 min with brefeldin A included in the appropriate washing solution. Emissions from membrane eYFP-ENaC were quantified using TIRF microscopy. Membrane ENaC levels were normalized to the membrane levels of the channel in the presence of Y-27632 just prior to washout.

Acute Activation of RhoA Signaling Rapidly Increases ENaC Activity—To better judge the ramifications of tight spatiotemporal control of ENaC by RhoA signaling, we directly monitored changes in channel activity over time upon relief of Rho kinase inhibition. Fig. 5A shows a summary graph of ENaC activity in the absence and presence of co-expression of Rho kinase CA with and without pretreatment with Y-27632. Augmentation of ENaC activity by Rho kinase CA was sensitive to Y-27632 with current density significantly decreasing in response to inhibitor.

Fig. 5B shows a summary of results from electrophysiological experiments mimicking the conditions of the TIRF microscopy experiments in Fig. 4. Cells transfected with mENaC and RhoAG14V were pretreated with 1 µM Y-27632 for 40 min.

membrane levels in the presence and absence of RhoAG14V. Reversing the fluorophores where eCFP-ENaC was expressed with eYFP-M in the presence and absence of RhoAG14V resulted in similar findings upon relief of Rho kinase inhibition (not shown).

In most cases (13 of 18) for cells expressing RhoAG14V and eYFP-tagged ENaC, it was possible to distinguish “hot spots” in the membrane where ENaC accumulated as a function of time following removal of Y-27632. Membrane eYFP emissions from such a cell before and 6, 12, and 18 min after washout are shown in Fig. 4C. These hot spots either represent defined membrane domains where vesicles containing ENaC dock and insert the channel into the membrane or represent vesicles containing ENaC approaching the membrane with intensity increasing as the vesicles near the membrane and fully occupy the field excited with TIRF microscopy. An alternative is that these hot spots represent domains where membrane ENaC accumulates prior to retrieval with RhoA signaling disrupting this retrieval.

Fig. 4D shows summarized results defining the time course of increases in membrane eYFP emissions upon washout of Y-27632 for cells expressing eYFP-tagged ENaC and RhoAG14V pretreated (closed squares; $n = 11$) and not pretreated (open squares; $n = 6$) with brefeldin A for 22 h. For these experiments, Y-27632 was added, as above, 40 min prior to washout with brefeldin A included in the appropriate bath and wash solutions. Cells pretreated with brefeldin A had significantly reduced levels of ENaC in the membrane following acute activation of RhoA signaling. As observed in control experiments (not shown; $n = 4$), relative ENaC membrane levels in cells co-transfected with RhoAG14V but not treated or washed of Y-27632 did not change over a 30-min period following 22 h of brefeldin A treatment. Because brefeldin A is an inhibitor of protein trafficking in the endomembrane system of mammalian cells (36) and counters the effects of RhoA on ENaC, these results, although not definitive, are consistent with RhoA promoting ENaC trafficking to the plasma membrane rather than retarding retrieval from this membrane.

Fig. 4E shows the time course of changes in channel activity over time upon relief of Rho kinase inhibition. Data points fit with an exponential rise to maximum equation. Open squares and circles show the change in relative membrane levels of eCFP-M in the absence and presence of RhoAG14V, respectively, upon washout of Y-27632.

Acute Activation of RhoA Signaling Rapidly Increases ENaC Activity—To better judge the ramifications of tight spatiotemporal control of ENaC by RhoA signaling, we directly monitored changes in channel activity over time upon relief of Rho kinase inhibition. Fig. 5A shows a summary graph of ENaC activity in the absence and presence of co-expression of Rho kinase CA with and without pretreatment with Y-27632. Augmentation of ENaC activity by Rho kinase CA was sensitive to Y-27632 with current density significantly decreasing in response to inhibitor.

Fig. 5B shows a summary of results from electrophysiological experiments mimicking the conditions of the TIRF microscopy experiments in Fig. 4. Cells transfected with mENaC and RhoAG14V were pretreated with 1 µM Y-27632 for 40 min.
RhoA signaling was acutely activated upon washout of Y-27632. Similar to its effects on ENaC activity in the presence of Rho kinase CA, Y-27632 significantly decreased ENaC activity in the presence of RhoAG14V from 600 ± 89 to 153 ± 20 pA/pF (not shown in the same figure; see also Fig. 1B). ENaC activity in cells pretreated with Y-27632 expressing RhoAG14V increased 20 and 40 min following washout of Y-27632. CHO cells were used for these experiments with voltage clamp conditions identical to Fig. 1.* versus mENaC alone; ** versus mENaC + Rho kinase CA. B, summary graph of current density for mENaC co-expressed with RhoAG14V in the presence of Y-27632, and 20 and 40 min after washout of Y-27632. CHO cells were used for these experiments with voltage clamp conditions identical to Fig. 1.* versus mENaC plus RhoAG14V (Fig. 1B); **, versus mENaC + RhoAG14V + Y-27632.

**FIGURE 5. Acute activation of RhoA signaling increases ENaC activity.** A, summary graph of the amiloride-sensitive current density for mENaC alone and with co-expression Rho kinase CA in the absence and presence of pretreatment with Y-27632 (1 µM, 40 min). CHO cells were used for these experiments with voltage clamp conditions identical to Fig. 1. *, versus mENaC alone; **, versus mENaC + Rho kinase CA. B, summary graph of current density for mENaC co-expressed with RhoAG14V in the presence of Y-27632, and 20 and 40 min after washout of Y-27632. CHO cells were used for these experiments with voltage clamp conditions identical to Fig. 1. *, versus mENaC plus RhoAG14V (Fig. 1B); **, versus mENaC + RhoAG14V + Y-27632.

**FIGURE 6. RhoA signaling affects endogenous ENaC activity in epithelia.** A, summary graph of recombinant ENaC activity in voltage-clamped CHO cells co-expressing RhoAG14V in the absence and presence of a cell-permeable, hybrid C3 exoenzyme (C2IN-C3) for 4 h. Voltage clamp conditions are identical to Fig. 1. B, summary graph of normalized (to before treatment) current across A6 epithelial cell monolayers treated with vehicle and C2IN-C3 for 4 h. Throughout these experiments, A6 cells were maintained in the presence of aldosterone.

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**RhoA Actions on ENaC Are Specific**—We wondered whether the actions of RhoA signaling on ENaC were specific or merely a manifestation of a general change in cellular trafficking. As a first step in addressing this question, we tested the effects of RhoA on Kv7.5. Standard protocols and solutions were used to measure activity of recombinant Kv7.5 expressed in CHO cells in the absence and presence of endogenous RhoA with C2IN-C3 in native A6 renal epithelial cells decreases Na⁺ reabsorption. It is well established that ENaC activity is limiting for Na⁺ reabsorption in these cells (22, 23). For these experiments, A6 cells were grown to confluence on permeable supports in the presence of aldosterone until they were avidly reabsorbing Na⁺. Cells were treated with vehicle and C2IN-C3 for 4 h with current measured before and 4 h after treatment.

**DISCUSSION**

The current results demonstrate that the small GTPases RhoA and K-Ras act independent of each other to enhance ENaC activity. Although the effects of RhoA and K-Ras on ENaC are similar in the sense that they both increase channel activity, their transduction pathways and mechanisms of ENaC activation are different. RhoA increases membrane levels of ENaC via a transduction pathway involving its downstream effectors Rho kinase and PI(4)P5K.

Rho-GTPases play regulatory roles in both cytoskeletal rearrangement and vesicle trafficking (38, 39). For instance, Cdc42, a Rho family GTPase, regulates targeting of secretory vesicles (40) and enhances exocytosis by mast cells (41, 42). Both RhoA and Rac1 have also been reported to regulate stimulus-induced exocytosis in endocrine, mast, and neuronal cells (43–45). Moreover, it was recently reported that growth factors, such as epidermal growth factor, induce translocation of functional TRPC5 channels to the plasma membrane through signaling involving the Rho-GTPase Rac1 (17). Rac1 was shown to be both necessary and sufficient to stimulate the rapid incorporation of functional TRPC5 channels into the plasma membrane through activation of PI(4)P5K. The current findings showing RhoA signaling rapidly increases membrane levels of functional ENaC support a similar transduction pathway and mechanism of action.

Rho-GTPases, as well as Rho kinase, activate PI(4)P5K, the enzyme that catalyzes the formation of PI(4,5)P₂, and thus, RhoA signaling potentially is a critical regulator of cellular...
PI(4,5)P2 pools (33, 46). We demonstrate here that PI(4)P5K is positioned between RhoA and ENaC in the signaling cascade leading to increased channel activity.

Phosphatidylinositides, particularly PI(4,5)P2, have a role in regulating the exocytic process. For instance, PI(4,5)P2 is required for vesicle docking or priming vesicles before release in neuroendocrine cells (47). In addition, many adaptors and targeting proteins, as well as cytoskeletal elements and molecular motors involved in vesicle movement directly or indirectly interact with PI(4,5)P2 (48–50). Thus, the membrane pool of PI(4,5)P2 may be important in both docking and fusion of vesicles. The current results suggest this to be the case for insertion of ENaC in response to RhoA signaling. We favor this mechanism over the alternatives that RhoA signaling slows retrieval or increases total cellular ENaC levels by blocking degradation or a retrieval pathway. We favor this mechanism because of the finding that Rho-GTPases via RhoA promote ENaC insertion in principal cells (51–53). Thus, RhoA signaling and its effects on ENaC are findings that Rho-GTPases via RhoA promote ENaC insertion, and this mechanism is particularly relevant for ENaC regulation in epithelial cells.

Measuring ENaC levels (8), and we demonstrate here that an inhibitor of trafficking in the endomembrane system, brefeldin A, abolishes the effects of RhoA on ENaC.

Regardless of mechanism, the experiments in Fig. 6 suggest that RhoA plays an important role in establishing membrane levels of ENaC in native epithelial cells. Currently, it is not clear whether this effect of RhoA is constitutive or regulated.

The pattern we observed for membrane ENaC expression in response to acute RhoA signaling is interesting. ENaC first appears in the membrane in defined regions or foci of just less than 1 μm in diameter. As mentioned under “Results,” these foci could represent one of three specialized membrane areas as follows: 1) vesicles docking with the membrane; 2) areas of the plasma membrane where ENaC is preferentially inserted; and 3) areas of the plasma membrane where ENaC is gathered prior to retrieval with RhoA signaling blocking the retrieval step.

Important questions raised by our findings are whether the rapid increase in functional ENaC in the membrane in response to RhoA signaling is specific for this channel or whether it is a more general mechanism of regulation. This is a difficult question to answer. The current results testing the effects of RhoA on Kv7.5 channels suggest that this regulation is specific for ENaC versus this latter channel. However, the commonality between Rac1 and RhoA regulation of TRPC5 and ENaC, respectively, suggests that it may also be a more general mechanism. Supporting a more general mechanism is the pervasive involvement of Rho-GTPases in vesicle movement and cytoskeleton regulation. Nevertheless, regulation of ENaC membrane levels by RhoA signaling could potentially provide a means for tight spatio-temporal control of Na+ entry into cells. This is of critical importance to epithelial cells responsible for mediated Na+ (re)absorption, because these cells are constantly challenged by large osmotic and trans-epithelial ion gradients and are potentially susceptible to Na+ overload. Supporting the potential physiological importance of RhoA regulation of ENaC are findings that Rho-GTPases via Rho kinase also modulate translocation of aquaporin-2 and the Na+/H+ exchanger to the apical plasma membrane of renal principal cells (51–53). Thus, RhoA signaling and its effects on transport proteins to include control of membrane ENaC levels may be part of a larger cellular program tightly controlling epithelial transport.

Acknowledgment—We thank Dr. LaGrange for critical reading of this manuscript and for making helpful comments.

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