Ras Activates the Epithelial Na\(^+\) Channel through Phosphoinositide 3-OH Kinase Signaling*

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Aldosterone induces expression and activation of the GTP-dependent signaling switch K-Ras. This small monomeric G protein is both necessary and sufficient for activation of the epithelial Na\(^+\) channel (ENaC). The mechanism by which K-Ras enhances ENaC activity, however, is uncertain. We demonstrate here that K-Ras activates human ENaC reconstituted in Chinese hamster ovary cells in a GTP-dependent manner. K-Ras influences ENaC activity most likely by affecting open probability. Inhibition of phosphoinositide 3-OH kinase (PI3K) abolished K-Ras actions on ENaC. In contrast, inhibition of other K-Ras effector cascades, including the MAPK and Ral/Rac/Rho cascades, did not affect K-Ras actions on ENaC. Activation of ENaC by K-Ras, moreover, was sensitive to co-expression of dominant negative p85\(^\text{PI3K}\). The G12C40 effector-specific double mutant of Ras, which preferentially activates PI3K, enhanced ENaC activity in a manner sensitive to inhibition of PI3K. Other effector-specific mutants preferentially activating MAPK and RalGDS signaling had no effect. Constitutively active PI3K activated ENaC independent of K-Ras with the effects of PI3K and K-Ras on ENaC not being additive. We conclude that K-Ras activates ENaC via the PI3K cascade.

Small monomeric G proteins, including Ras, are GTP-dependent signaling switches that control cell growth, proliferation, and differentiation, as well as playing important signaling roles in differentiated cells (1–3). There are four homologous Ras proteins, K-RasA, K-RasB, H-Ras, and N-Ras, capable of activating similar downstream effectors with Ras having three primary first effectors, Raf, Raf/GDS, and PI3K,\(^1\) initiating the MAPK and Ral/Rac/Rho cascades, did not affect K-Ras actions on ENaC. Activation of ENaC by K-Ras, moreover, was sensitive to co-expression of dominant negative p85\(^\text{PI3K}\). The G12C40 effector-specific double mutant of Ras, which preferentially activates PI3K, enhanced ENaC activity in a manner sensitive to inhibition of PI3K. Other effector-specific mutants preferentially activating MAPK and RalGDS signaling had no effect. Constitutively active PI3K activated ENaC independent of K-Ras with the effects of PI3K and K-Ras on ENaC not being additive. We conclude that K-Ras activates ENaC via the PI3K cascade.

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\(^1\) The abbreviations used are: PI3K, phosphoinositide 3-OH kinase; MAPK, mitogen-activated protein kinase; ENaC, epithelial Na\(^+\) channel; CHO, Chinese hamster ovary; EV, evanescent field; P\(_E\), cochlear partition; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; CFP-M, CFP-tagged membrane marker; GDP\(_\alpha\)S, guanyl-5’-y1 thiphosphate; NHS-LC, succinimidyl-6-(biotinamido) hexanoate.

β-subunit of L-type Ca\(^2+\) channels promoting retrieval from the plasma membrane and decreased channel activity (5–7); Rac and Rho have opposing actions on ether-a-go-go-related gene K\(^+\) channels with the prior rapidly activating the channel and latter quickly decreasing channel activity (8); similarly, Rap and Ras have opposing actions on muscarinic K\(^+\) channels (9); and Ras via MAPK signaling decreases the activity of IRK1 by promoting retrieval of the channel from the plasma membrane (10) but increases the activity of T-type Ca\(^2+\) channels (11). Thus, a wide variety of ion channels are final effectors for Ras and other small G proteins.

Activity of the epithelial Na\(^+\) channel (ENaC) is rate-limiting for electrogenic Na\(^+\) transport across electrically tight epithelia, such as that lining the distal colon and renal nephron (12–14). Systemic Na\(^+\) levels are maintained in balance through a classic negative feedback pathway involving activation of ENaC by the steroid hormone aldosterone. Similar to other steroids, aldosterone affects gene expression to influence the activity of its target proteins; however, aldosterone-dependent increases in ENaC activity precede effects on channel expression leading to the proposal that aldosterone influences ENaC activity by promoting the expression of mobile signaling molecules capable of transducing information from the nucleus to existing channels. K-RasA is regulated by aldosterone at the level of transcription (15) with aldosterone promoting MAPK signaling in epithelia via induction of this small G protein (16, 17). Moreover, K-RasA is necessary and sufficient for ENaC activation in some models (18, 19), which makes this small G protein an attractive candidate to transduce aldosterone actions from the nucleus to the channel. However, the direct role of Ras in regulation of ENaC is unclear with its mechanism of action and the cell signal transduction pathways involving Ras actions on ENaC being uncertain.

Insulin, in addition to aldosterone, increases Na\(^+\) reabsorption with PI3K being necessary to both aldosterone and insulin actions on Na\(^+\) transport (20–24). Moreover, aldosterone increases the levels of the phospholipid products of PI3K in epithelial cells, and these phospholipids have recently been shown to directly enhance ENaC activity (20, 25, 26). Because PI3K is a first effector of Ras, we tested whether activation of ENaC in response to K-Ras was mediated by signaling through this lipid kinase. The current results are consistent with K-Ras activating ENaC via PI3K.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of reagent grade and were purchased from Calbiochem, BioMol (Plymouth Meeting, PA), or Sigma, unless noted otherwise. The mammalian expression vectors encoding ENaC subunit cDNAs have been described previously (24, 26, 27). Expression vectors encoding hemagglutinin-tagged wild type, constitutively active (G12V), and dominant negative (S17N) K-Ras were from the Guthrie Research Institute. Expression vectors encoding Ras effector-specific
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Results

K-Ras Increases ENaC Activity in a GTP-dependent Manner

To investigate the actions of K-Ras on ENaC, we reconstituted the channel in CHO cells in the absence and presence of co-expressed wild type, constitutively active (G12V) and dominant negative (S17N) K-Ras. Human ENaC was reconstituted by co-expressing α, β and γ channel subunits together. Fig. 1A shows typical currents from a voltage clamp experiment performed on a CHO cell containing human ENaC before and after treatment with amiloride. Amiloride is an open channel blocker of ENaC (14). Currents were elicited by applying test pulses from 60 to −100 mV with 20-mV steps. Overexpression of ENaC resulted in robust amiloride-sensitive inward Na+ currents that were not present in untransfected cells (not shown; see Refs. 24, 26, and 27). Fig. 1B shows ENaC currents before and after treatment with amiloride in a cell expressing the channel alone (left) and in a cell expressing both the channel and constitutively active K-Ras (right). Currents were elicited by voltage ramping from 60 mV down to −100 mV (holding potential = 30 mV). As summarized in Fig. 1C, co-expression of wild type and constitutively active but not dominant negative K-Ras significantly increased ENaC activity. Overexpression of K-Ras had no effect on cell capacitance as shown in Fig. 1D. We interpret these results as showing that K-Ras enhances ENaC activity, but that this channel does have some basal activity in CHO cells that is independent of K-Ras signaling.

We next tested whether K-Ras actions on ENaC were dependent on GTP activation of this small G protein. Fig. 2A shows current through ENaC in the presence of constitutively active K-Ras elicited by a train of voltage ramps applied every 5 s over the course of several min. For this experiment, 2.0 mM GDPβS replaced the GTP in typical pipette solutions. As the cytosol was dialyzed with GDPβS, ENaC activity decreased over time indicating that GTP was necessary for K-Ras to enhance channel activity. Run-down of ENaC activity in whole cell voltage clamp experiments was never observed (not shown, see Refs. 26 and 27), and thus the decrease in activity observed in Fig. 2A resulted from GDPβS-competing GTP. Fig. 2B shows an overlay of ENaC currents before and after dialyzing GDPβS and the subsequent application of amiloride. As demonstrated by the summary graph in Fig. 2C, GTP is necessary for K-Ras activation of ENaC. Interestingly but not further pursued in the current study, we saw a modest effect of GDPγS on the K-Ras-independent basal activity of ENaC suggesting that other endogenous GTP-dependent proteins also impinge upon activity of this channel.

K-Ras Activation of ENaC Is Mediated by PI3K Signaling

We next asked whether K-Ras activated ENaC through signaling via one of its downstream effector cascades, the MAPK, PI3K, and Rac/Ral/Rho cascades. Fig. 3A shows overlays of ENaC currents from typical whole cell voltage clamp experiments elicited by standard voltage ramps before and after amiloride. Currents are from cells expressing ENaC plus K-Ras in the absence (top) and presence (bottom) of pretreat-

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2 A. Staruschenko, P. Patel, Q. Tong, J. L. Medina, and J. D. Stockand, unpublished observations.
**Fig. 1. K-Ras enhances ENaC activity.** A, typical macroscopic currents before and after amiloride under voltage clamp conditions from a CHO cell transfected with all three human ENaC subunits with currents elicited by test pulses with 20 mV steps from −60 to −100 mV from a holding potential of 30 mV. B, representative macroscopic currents before and after amiloride under voltage clamp conditions from CHO cells transfected with all three human ENaC subunits in the absence (left) and presence of co-expression of constitutively active K-Ras (right) with currents elicited by voltage ramps from 60 to −100 mV from a holding potential of 30 mV. C, summary graph of the mean ± S.E. amiloride-sensitive current density at −80 mV for voltage-clamped CHO cells expressing human ENaC in the absence and presence of wild type (K-Ras), constitutively active (G12V), and dominant negative (S17N) K-Ras. The number of observations in each group is shown. *, \( p < 0.05 \) versus ENaC alone group. D, whole cell capacitance for the groups shown in C. hENaC, human ENaC.

**Fig. 2. K-Ras activates ENaC in a GTP-dependent manner.** A, typical time course of the actions of dialyzing intracellular GTP with GDPβS in cells expressing ENaC plus constitutively active K-Ras. Shown are macroscopic currents under voltage clamp conditions elicited by a continuous train of 500-ms voltage ramps (60 to −100 mV from a holding potential of 30 mV) applied every 5 s over the course of the experiments. For this experiment pipette GTP was replaced with GDPβS, and amiloride was applied to the extracellular bath solution at the end of the experiment. B, an overlay of three macroscopic currents from the experiment described in A before and after dialyzing GDPβS and then applying amiloride to the extracellular bath solution. C, summary graph of the amiloride-sensitive current density for ENaC in the absence and presence of wild type, constitutively active, and dominant negative K-Ras before and after dialyzing intracellular GTP with GDPβS. Also shown are the current densities for ENaC in the absence and presence of constitutively active K-Ras with GTP in the pipette. *, \( p < 0.05 \) versus beginning current densities before dialysis of GDPβS. hENaC, human ENaC.
Fig. 3. K-Ras activates ENaC via PI3K. A. overlays of representative macroscopic currents elicited by voltage ramps before and after amiloride under voltage clamp conditions from CHO cells transfected with ENaC plus K-Ras without (top) and with (bottom) pretreatment with the PI3K inhibitor wortmannin (200 nM, 5 h.). B. summary graph of current density for ENaC in the absence and presence of wild type K-Ras with and without pretreatment with inhibitors of MAPK (Mek1/2 inhibitors PD98059 and U0126), Raf/Rac/Rho (Rho kinase inhibitor Y27632, Rho inhibitor C3 exoenzyme included in pipette solution), and PI3K (wortmannin) signaling. *, p < 0.05 for ENaC plus K-Ras versus ENaC alone; **, for ENaC plus K-Ras + wortmannin versus ENaC plus K-Ras. hENaC, human ENaC.

Results shown in Fig. 5 are also consistent with PI3K being necessary for K-Ras activation of ENaC. Fig. 5A shows a summary graph of ENaC activity when channel subunits are expressed alone and with K-Ras in the absence and presence of co-expression of dominant negative PI3K (p85 ΔSH2-C) (3). Inclusion of dominant negative PI3K with K-Ras significantly decreased ENaC activity from 445 ± 12 to 317 ± 38 pA/pF. Fig. 5B–D, shows population histograms describing ENaC activity in the absence of K-Ras (Fig. 5B), and presence of co-expressed K-Ras (Fig. 5C) and co-expressed K-Ras plus dominant negative PI3K (Fig. 5D). The populations describing both ENaC alone and ENaC in the presence of K-Ras were normally distributed around a single mean (ENaC alone = 156 ± 11 pA/pF). In contrast, two ENaC populations were observed when the channel was co-expressed with K-Ras plus dominant negative PI3K. These populations were normally distributed around means of 155 ± 14 (n = 13) and 479 ± 36 (n = 13) pA/pF. Two mechanisms may explain the appearance of the population with the lower activity when ENaC is co-expressed with both K-Ras and dominant negative PI3K. It may result from a titration effect or from the dominant negative PI3K uncoupling K-Ras signaling to the channel. Results shown in Figs. 3 and 4 support the latter possibility. Moreover, ENaC activity in the presence of co-expressed K-Ras is normally distributed, indicating that in this population there was no titration effect. Finally, when ENaC was co-expressed with K-Ras plus another construct that did not impact PI3K signaling, we saw a single distribution with a mean current density of 433 ± 53 pA/pF (n = 15, not shown), which was similar to that observed with K-Ras alone, excluding a titration effect. Thus, we interpret the results in Fig. 5D as demonstrating that dominant negative PI3K uncouples K-Ras to ENaC signaling. If this interpretation is correct, then there must be a threshold effect of PI3K on the
channel with activated PI3K resulting in channels with high activity and inactivated PI3K leading to channels even in the presence of K-Ras with basal activity. This possibility is consistent with our previous findings that the phospholipid products of PI3K directly affect ENaC by stabilizing channel gating transitions (26). We predicted that if K-Ras activated ENaC via PI3K, then expression of constitutively active PI3K alone with ENaC should activate the channel, and the effects of co-expressing PI3K with K-Ras on ENaC should not be additive. Indeed, co-expression of constitutively active PI3K (myr-PI3K) with K-RasG12V (571/H11006) did not show an additive effect on ENaC activity when the channel was co-expressed with the C40 mutant. Shown here is a representative current overlay before and after amiloride.

FIG. 4. The PI3K Ras effector-specific mutant activates ENaC. A, overlays of representative macroscopic currents elicited by voltage ramps before and after amiloride (amil) for ENaC plus Ras effector-specific mutants: G12:E38 (c-Raf kinase, MAPK signaling), G12:G37 (RalGDS, Rap/Rac/Rho signaling), and G12:C40 (PI3K). The effects of pretreating with wortmannin were also tested on ENaC activity when the channel was co-expressed with the C40 mutant. Shown here is a representative current overlay before and after amiloride. B, summary graph of current density for ENaC in the absence and presence of constitutively active Ras and the constitutively active effector-specific mutants of Ras. *, p < 0.05 versus ENaC alone. C, summary graph of the amiloride-sensitive current density for ENaC in the absence and presence of constitutively active PI3K effector-specific C40 Ras mutant with and without pretreatment with wortmannin. *, p < 0.05 versus ENaC; **, versus ENaC plus RasG12C40; hENaC, human ENaC.
different in the presence of constitutively active K-Ras, although activity is increased (see Fig. 1). The results in Figs. 6 and 7, considering those in Figs. 1–5 and our past publications (18, 26), are most consistent with K-Ras via PI3K impinging upon ENaC open probability.

**DISCUSSION**

The results of the current study demonstrate that K-Ras activates ENaC in a GTP-dependent manner with PI3K being necessary for this activation. Moreover, that ENaC is activated by the PI3K-specific effector mutant of Ras, RasG12C40, in a wortmannin-sensitive manner is consistent with PI3K being functionally positioned between K-Ras and the channel. Such a transduction pathway fits well with PI3K being a known first effector of K-Ras (3) and with observations showing that both K-RasA and PI3K are necessary for aldosterone actions on ENaC and Na\(^+\)/H\(^+\) transport, respectively, in native epithelia (18, 20, 21, 35).

Overexpression of constitutively active K-Ras with ENaC in X. laevis oocytes was previously shown by Verrey and colleagues (19) to result in little change in channel activity in the face of decreasing membrane area and surface expression of ENaC suggesting that K-Ras activated ENaC by increasing open probability. In the current study, we detected K-Ras-dependent increases in ENaC activity without changes in membrane area (capacitance, Fig 1). That ENaC activity increased with no change in membrane area simplifies the interpretation of these results leading to a conclusion similar to Verrey's group (19) that K-Ras activates ENaC. This conclusion is also consistent with our previous findings demonstrating that K-RasA and proper posttranslational modification of this small G protein are necessary for aldosterone to increase ENaC open probability in native A6 epithelial cells (18, 36–38). The finding reported here that GTP is required for K-Ras actions on ENaC (Fig. 2) is novel and suggests that K-Ras acting in a classic manner regulates ENaC as a GTP-dependent signaling switch.

Similar to K-RasA, PI3K is also necessary for aldosterone to increase Na\(^+\) reabsorption in renal epithelia (20, 21, 23, 24, 35). Moreover, the two aldosterone-induced proteins, K-RasA and Sgk, are part of a converging signaling cascade with PI3K positioned between them impinging upon Na\(^+\) reabsorption (24). Aldosterone via Sgk is believed to increase the half-life of ENaC resident to the plasma membrane by retarding channel retrieval mediated by the ubiquitin ligase Nedd4-2 (23, 32–34).

The results in Figs. 3–7 are consistent with K-Ras via PI3K enhancing ENaC activity with these effects most likely being attributable to increases in channel open probability and not because of actions on channel number. Recently, we (26) as well as other groups (25) have demonstrated that the phospholipid products of PI3K directly enhance ENaC open probability in excised patches. Thus, PI3K is a central signaling intermediary in transduction cascades that increase both the open probabili-
We, similar to other investigators interested in aldosterone signaling to ENaC, accept the likelihood that aldosterone affects ENaC through multiple transduction cascades; however, the current results in consideration of our previous findings and those published by others, position us to propose a completed linear transduction cascade whereby aldosterone impacts ENaC activity. This cascade would be initiated by increased transcription of K-RasA in response to trans-activation of ENaC and the number of channels in the plasma membrane.

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by the aldosterone-mineralocorticoid receptor complex binding to a cis-acting steroid response element in the K\textsuperscript{+} channel gene and subsequent increases in the GTP-complexed active levels of K-Ras via mass action, activation of PI3K by GTP-activated K-RasA, production of phospholipids by this activated lipid kinase, and dependent activation of ENaC by these phospholipid products increasing channel open probability. This linear cascade likely is just one branch of a larger non-linear branching cascade containing several distinct signaling intermediates and points of divergence and convergence with ENaC activity ultimately reflecting the combined sum of all signaling inputs to include the linear K-RasA-PI3K-ENaC cascade.

Thus, ENaC, similar to other ion channels such as IRK1 and ether-a-go-go-related gene K\textsuperscript{+} channels and L- and T-type Ca\textsuperscript{2+} channels (4–11), is a final effector of a signaling cascade initiated by a small G protein. We demonstrate here that K-Ras enhances ENaC activity via PI3K signaling. This is the first report (of which we are aware) that shows that Ras modulates ENaC activity via PI3K.

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