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Ras couples phosphoinositide 3-OH kinase to the epithelial Na^+ channel

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Abstract

Aldosterone induces the expression of the small G protein K-Ras. Both K-Ras and its 1st effector phosphoinositide 3-OH kinase (PI3-K) are necessary and sufficient for the activation of ENaC increasing channel open probability. The cell signaling mechanism by which K-Ras enhances ENaC activity, however, is uncertain. We demonstrate here that K-Ras significantly activates human ENaC reconstituted in Chinese hamster ovary cells ~3-fold. Activation in response to K-Ras was sensitive to the irreversible PI3-K inhibitor wortmannin but not the competitive LY294002 inhibitor of this phospholipid kinase. Similarly, a PI3-K 1st effector-specific Ras mutant (G12:C40) enhanced ENaC activity in a wortmannin but not LY294002 sensitive manner. Constitutively active PI3-K also enhanced ENaC activity but in a wortmannin and LY294002 sensitive manner with the effects of PI3-K and K-Ras not being additive. The activation of ENaC by PI3-K was also sensitive to intracellular GDPβS. Constitutively active PI3-K that is incapable of interacting with K-Ras (K227E p110α) acted as dominant negative with respect to the regulation of ENaC even in the presence of K-Ras. K-Ras is known to directly interact with PI3-K with aldosterone promoting this interaction. Here we demonstrate that K-Ras also interacts with ENaC through an, as yet, undetermined mechanism. We conclude that K-Ras enhances ENaC activity by localizing PI3-K near the channel and stimulating of PI3-K activity.

Keywords: K-Ras; Phosphoinositide 3-OH kinase; ENaC activation

1. Introduction

The activity of the amiloride-sensitive epithelial Na^+ channel (ENaC) is rate limiting for Na^+ (re)absorption across electrically tight epithelia, such as that lining the renal distal nephron [1–3]. Genetic mutations in humans leading to gain and loss of ENaC function, as well as aldosterone release and end organ effectiveness cause abnormalities in electrolyte and water balance and thus, aberrant blood pressure control [4]. Aldosterone maintains systemic Na^+ balance by enhancing ENaC activity. Aldosterone, similar to other steroids, impinges upon cellular activity, including control of Na^+ reabsorption in the distal nephron, by modulating gene expression [1]. Though ENaC activity is increased, aldosterone-dependent increases in Na^+ reabsorption do not result, at least initially, from effects on ENaC gene expression. This has led to the hypothesis that aldosterone enhances ENaC activity by promoting the expression of mobile signaling molecules capable of transducing information from the nucleus to existing channels. One such aldosterone-induced signaling molecule that is necessary and sufficient for ENaC activation in some epithelia is the small, monomeric G protein K-Ras [5–9]. The mechanism by which K-Ras affects ENaC is uncertain.

Similar to K-Ras, phosphoinositide 3-OH kinase (PI3-K) is necessary for Na^+ reabsorption and aldosterone action [10–14]. Aldosterone, moreover, increases the levels of the phospholipid products of PI3-K in epithelial cells and these phospholipids have recently been shown to directly enhance ENaC activity [10,15,16]. In addition to the serine/threonine kinase c-Raf and the GDP/GTP exchange factor RalGDS, PI3-K is a 1st effector that directly interacts with K-Ras [17–19]. Recent evidence suggests that aldosterone promotes K-Ras-PI3-K interaction in epithelia [14]. These results led us to test here whether the activation of ENaC in response to K-Ras is mediated by signaling through PI3-K. The current results are consistent with K-Ras activating

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ENaC via PI3-K with the small G protein coupling activated PI3-K to the channel.

2. Materials and methods

2.1. Materials

All chemicals were from Calbiochem (San Diego, CA), BioMol (Plymouth Meeting, PA) or Sigma (St. Louis, MO) unless noted otherwise. Phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and histone carries were from Echelon Biosciences Incorp. (Salt Lake City, UT). The mammalian expression vectors encoding ENaC subunit cDNAs have been described previously [14,16,20]. The expression vectors encoding HA-tagged K-Ras and its constitutively active mutant (K-Ras G12V) were from the Guthrie Research Institute. The expression vectors encoding K227E p110α PI3-K and the Ras PI3-K-1st effector specific Ras double mutant G12:C40 were from J. Downward and have been described previously [19,21–23]. The expression vector encoding membrane targeted constitutively active PI3-K (pUSE-amp-p110α) was from Upstate Biotech (Lake Placid, NY). This plasmid encodes p110α fused to an amino-terminal Src myristoylation sequence, which localizes PI3-K to the plasma membrane, where it is then constitutively active [24]. Materials used in the Western blot analysis were from Bio-Rad (Hercules, CA). GST-Ras agarose was from Upstate Biotech. (Lake Placid, NY). The mouse monoclonal anti-myc antibody was from Clontech (Palo Alto, CA). The mouse monoclonal anti-HA antibody and rat monoclonal anti-HA agarose were from Roche (Indianapolis, IN). Anti-mouse HRP conjugated 2° antibodies were from Kirkegaard-Perry Laboratories (Gaithersburg, MD). ECL reagents were from PerkinElmer Life Sciences (Boston, MA). CHO cells were maintained with standard culture conditions (DMEM+10% FBS, 37 °C, 5% CO_2) and transfected using the Polyfect reagent (Qiagen; Valencia, CA) as described previously [14,16,20].

2.2. Electrophysiology

Whole-cell macroscopic current recordings of ENaC reconstituted in CHO cells were made under voltage-clamp conditions with constant perfusion using standard methods [14,16,20]. Similarly, recordings of current through ENaC in excised, outside-out patches made from CHO cells expressing the channel were acquired with standard methods [16,25]. In both cases the extracellular bath and intracellular pipette solutions were (in mM) 160 NaCl, 1 $CaCl_2$, 2 $MgCl_2$ and 10 HEPES (pH 7.4); and 120 CsCl, 5 NaCl, 5 EGTA, 2 $MgCl_2$, 2 ATP, 0.1 GTP, 10 HEPES (pH 7.4), respectively. In some experiments, 2.0 mM GDPβS replaced the 0.1 mM GTP in pipette solutions. Macroscopic current recordings were acquired with an Axopatch 200B (Axon Instr.; Union City, CA) interfaced via a Digidata 1322A (Axon Instr.) to a PC running the pClamp 9 suite of software (Axon Instr.). All currents were filtered at 1 kHz. Both a family of test pulses (500 ms each) stepping by 20 mV increments from a holding potential of 30 mV to 60 mV to −100 mV and voltage ramps (300 ms) from 60 mV to −100 mV were used to generate current–voltage (I–V) relations and to measure ENaC activity at −80 mV. Whole-cell capacitance was routinely compensated and was approximately ~8 pF for CHO cells. Series resistances, on average 2–5 MΩ, were also compensated. Current recordings of ENaC in excised, outside-out patches were acquired with a PC505B patch clamp amplifier (Warner Instr.) interfaced via a Digidata 1322A (Axon Instr.) to a PC running the pClamp 9 suite of software (Axon Instr.). Gap-free current recordings were made at 0 mV.

For experiments testing PIP_3 on ENaC, water soluble, short-chain (dioctanoyl) PIP_3 was prepared in aqueous stocks at 1 mM by sonication for 30 min and stored at −70 °C as described previously [16]. Stock phospholipids were mixed just prior to use with an equal volume of a carrier solution containing histone H1 (0.2 mM) and sonicated again for 10 min. Sonicated phospholipid/carrier was then applied to the bathing solution of outside-out patches.

2.3. Biochemistry

Immunoprecipitation experiments were performed using standard procedures described previously [6,8,20,26,27]. In brief, cells co-expressing myc-tagged ENaC and HA-tagged K-Ras were lysed in gentle lysis buffer (1.0% NP-40) with lysates being cleared and normalized for total protein concentration. Whole-cell lysates (400 μl at 1 μg/μl total protein) were treated with anti-HA agarose overnight at 4 °C. Immunoprecipitants were washed 3 times with 400 μl gentle lysis buffer and resuspended in Lamellia sample buffer and 20 mM DTT, heated at 85 °C for 10 min, run on 7.5% poly-acrylamide gels in the presence of SDS, transferred to nitrocellulose and probed with antibody in tris-buffered saline supplemented with 5% dried milk (Nestle; Solon, OH) and 0.1% Tween-20.

For GST pull-down experiments, CHO cells were transfected with myc-tagged ENaC and lysed in gentle lysis buffer. Whole cell lysates (400 μl at 1 μg/μl) were exposed to GST-Ras agarose (25 μg) overnight at 4 °C with constant agitation. Proteins binding GST-Ras agarose were precipitated, washed 3 times with gentle lysis buffer, resuspended in sample buffer, separated with SDS-PAGE and transferred to nitrocellulose. Western blots containing precipitated proteins were probed as described above.

The Western blot analysis of Akt and phospho-Akt was performed using standard procedures [6]. In brief, CHO cells were extracted in gentle lysis buffer in the presence of phosphatase inhibitors. Western blots containing equal amounts of total protein (~100 μg) per lane were probed with anti-Akt and anti-phospho-Akt antibodies (Cell Signaling; Beverly, MA).

2.4. Statistics

All patch clamp data are presented as mean±S.E. Data were compared with a *t*-test with $P<0.01$ considered significant.

3. Results

We reconstituted human ENaC in CHO cells to test the effects of K-Ras on channel activity. ENaC was reconstituted by co-expressing α,β,γ-hENaC subunits as previously described [14,16,20]. Channel activity was assessed as the amiloride-sensitive inward Na^+ current at −80 mV under voltage-clamp conditions. Fig. 1 shows the macroscopic currents from representative whole cell experiments elicited by a family of test pulses stepping from a holding potential of 30 mV to 60 mV to −100 mV. These cells expressed ENaC in the absence (A) and presence (B) of the co-expression of K-Ras. Currents before (top) and after (bottom) treatment with the ENaC inhibitor amiloride are shown. As reported previously, CHO cells contain no endogenous, amiloride-sensitive ENaC current [14,16,20]. The current–voltage (I–V) relations for macroscopic ENaC currents in the absence and presence of K-Ras are shown in Panel C. As summarized in Panel D, the co-expression of K-Ras significantly increased ENaC activity from 1.2±0.10 to 3.6±0.2 nA. K-Ras had no effect on cell capacitance, which was ~8 pF (not shown).

Since PI3-K is a 1st effector of K-Ras [19] and both K-Ras and PI3-K are necessary for aldosterone-stimulation of ENaC [6,8,10,14,28] with this corticosteroid promoting their association in epithelia [14], we asked whether PI3-K was also necessary for K-Ras activation of ENaC. Fig. 2 summarizes ENaC activity at −80 mV in cells expressing both the channel and K-Ras before and after pretreatment with two distinct PI3-K inhibitors wortmannin (0.2 μM, 5 h) and LY294002 (50 μM, 5 h). Wortmannin at this concentration is specific for PI3-K and is an irreversible inhibitor that covalently modifies the phospholipid kinase [29]. Similarly, at this dose, LY294002 is a specific inhibitor of PI3-K functioning in a competitive manner [30]. As shown, wortmannin but not LY294002 significantly decreased ENaC activity in the presence of K-Ras. Neither inhibitor affected cell capacitance (not shown).

The lack of an effect of LY294002 on K-Ras augmented ENaC activity led us to test whether this PI3-K inhibitor was indeed effective under our experimental conditions. Fig. 2B shows a representative (n=2) Western blot analyzing the actions of LY294002 and wortmannin on the activation of the PI3-K effector Akt [31]. For these experiments the active (phosphorylated; top blot) and total (bottom blot) levels of Akt were assessed in the absence (control) and presence of co-expressed active PI3-K with and without pretreatment with LY294002 (50 μM) and wortmannin (0.2 μM) for 2 h. In contrast to the lack of a LY294002 effect on K-Ras augmented ENaC activity, both inhibitors clearly blocked PI3-K activation of Akt.

To more fully understand the regulation of ENaC by K-Ras and its 1st effector PI3-K, we further probed the actions of this latter kinase on the channel. Fig. 3 shows a summary graph of ENaC activity in the absence and presence of co-

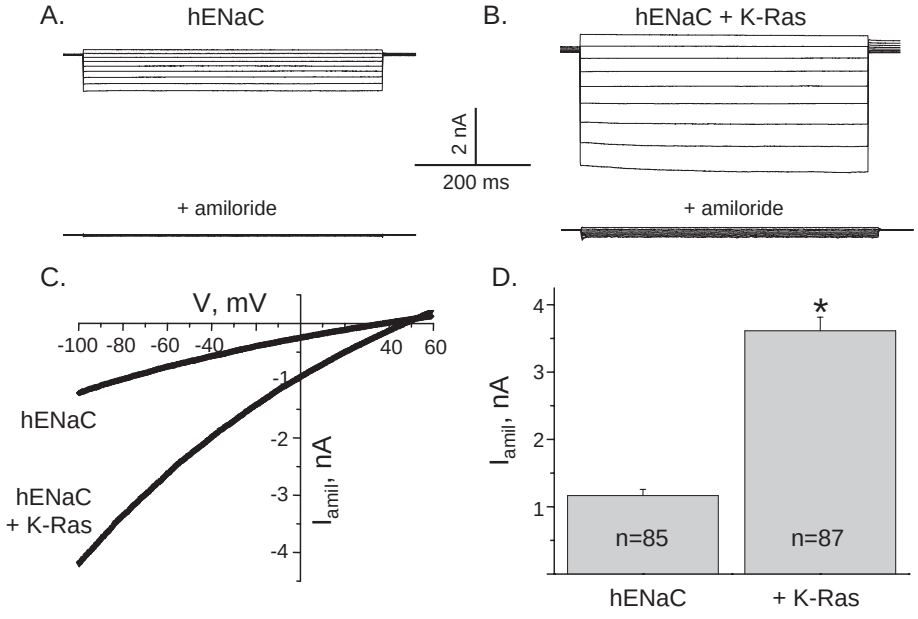

Fig. 1. K-Ras augments ENaC activity. Typical macroscopic currents elicited by voltage steps from 60 to −100 mV in voltage clamped CHO cells expressing ENaC alone (A) and with K-Ras (B) before (top) and after (bottom) amiloride. (C) Current–voltage relation of macroscopic, amiloride-sensitive ENaC currents in CHO cells expressing ENaC alone and with K-Ras. (D) Summary graph of amiloride-sensitive ENaC current in cells expressing the channel alone and with K-Ras. *P vs. hENaC alone.

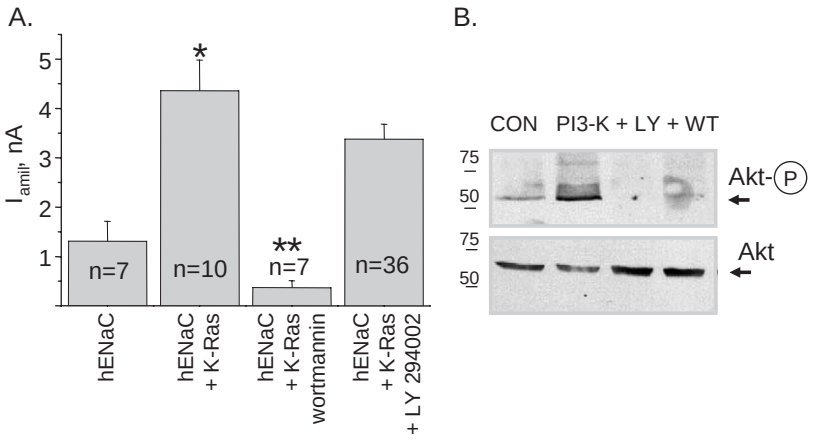

Fig. 2. PI3-K is necessary to K-Ras activation of ENaC. (A) Summary graph of amiloride-sensitive ENaC currents in cells expressing the channel alone and with K-Ras before and after wortmannin and LY294002. *P vs. hENaC alone, **vs. hENaC+K-Ras, hENaC+K-Ras+LY294002 n.s. vs. hENaC+K-Ras. (B) Representative Western blots (n=2) containing whole cell lysates from untransfected control CHO cells and those over-expressing constitutively active myristoylated PI3-K in the absence and presence of treatment with LY294002 and wortmannin. Both blots contain the same lysate with top and bottom probed with anti-phospho-Akt and anti-Akt antibodies, respectively.

expression of constitutively active PI3-K (myristoylated-p110α) with and without pretreatment with wortmannin (0.2 μM, 5 h) and LY294002 (50 μM, 5 h). Also shown are the effects on ENaC activity of co-expressing constitutively active PI3-K with constitutively active K-Ras. Unlike K-Ras, the enhancement of ENaC activity by constitutively active PI3-K was sensitive to both wortmannin and LY294002. In addition, K-Ras and PI3-K were not additive with respect to activating ENaC.

Since K-Ras and PI3-K were not additive with respect to increasing ENaC activity and, at least, one inhibitor of PI3-K blocked activation of the channel by this small GTPase, we tested whether PI3-K might be downstream of K-Ras between this small G protein and the channel. To begin testing this hypothesis, we asked whether GTP hydrolysis was necessary for increased ENaC activity in the presence of constitutively active PI3-K. Our rationale is that for K-Ras to interact with its 1st effector, it must be in its active, GTP-bound state [22,23]. Fig. 4A shows I–V relations for macroscopic currents in a typical voltage-clamped CHO cell expressing the channel plus constitutively active PI3-K before (CON) and after (3 min) dialyzing the intracellular solution with GDPβS (2 mM) to impede GTP hydrolysis by GTPases. Amiloride was subsequently added to the extracellular bathing solution at the end of each experiment (amil). These experiments were performed in a paired manner and thus, the I–V curves are from the same cell. Shown in Fig. 4B is a summary graph (n=10) showing the effects on ENaC activity in the presence of active PI3-K of dialyzing GDPβS into the intracellular fluid. Dialyzing GDPβS into the intracellular fluid markedly and quickly decreased ENaC activity even in the presence of activated PI3-K. The time-course of this effect suggests a close link between GTP hydrolysis and PI3-K augmentation of ENaC activity for the time-course of this action is short approximating the diffusion rate of GDPβS from the pipette solution into the cell.

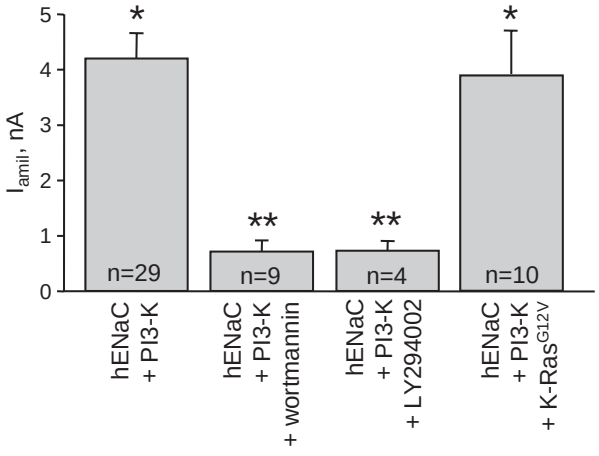

Fig. 3. K-Ras and PI3-K are part of the same signaling cascade to ENaC. Summary graph of amiloride-sensitive ENaC currents in cells expressing hENaC+constitutively active myristoylated PI3-K in the absence and presence of wortmannin and LY294002. Also shown is the current when hENaC is co-expressed with both constitutively active myristoylated PI3-K and constitutively active K-Ras. *P vs. hENaC alone (from Fig. 1), **vs. hENaC+PI3-K, hENaC+PI3-K+K-Ras n.s. vs. hENaC+PI3-K.

To further test the idea that K-Ras activates ENaC via PI3-K signaling, we co-expressed with ENaC, a 1st effector specific mutant of Ras (G12:C40) that only has the capacity to interact with and thus activate PI3-K [21–23]. This effector specific Ras mutant does not activate other Ras 1st effectors, such as c-Raf and Ral.GDS. As shown in Fig. 5, Ras G12:C40, similar to K-Ras, significantly increased ENaC activity. Also similar to K-Ras, but different from constitutively active myristoylated-p110α PI3-K, the activation of ENaC by this 1st effector specific Ras mutant was sensitive to pretreatment with wortmannin (0.2 μM, 5 h) but not LY294002 (100 μM, 5 h). Also similar to K-Ras, the C40 Ras mutant had no effect on cell capacitance (not shown).

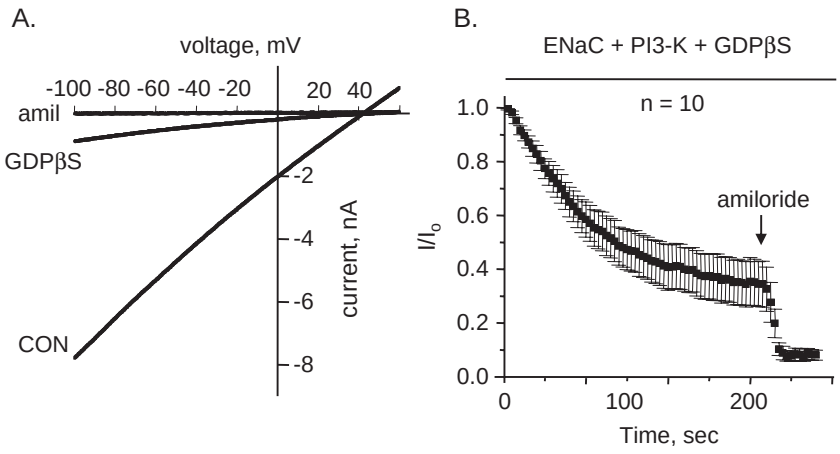

Fig. 4. Activate G-protein signaling is necessary for PI3-K activation of ENaC. (A) Typical macroscopic I–V relations for a voltage-clamped CHO cell expressing ENaC plus constitutive active myristoylated PI3-K before (con) and after dialyzing intracellular GTP with GDPβS and subsequent addition of amiloride. Currents were sequentially acquired from the same cell after treatment and were elicited by voltage ramps. (B) Summary graph of relative ENaC activity showing the time course of inhibition of PI3-K stimulated ENaC by dialysis of intracellular GTP with GDPβS. Subsequent addition of amiloride noted with arrow. Mean±S.E. for 10 experiments shown.

Results in Figs. 6 and 7 are from experiments testing the idea that in addition to activating PI3-K to increase ENaC activity, K-Ras physically localized PI3-K near the channel. We asked first whether K-Ras and ENaC interact with each other. Fig. 6A shows results from a typical in-vitro GST-pull-down experiment (n=4) where whole cell lysate from cells expressing eGFP, myc-tagged αENaC, a myc-tagged control protein, and myc-tagged α, β and γENaC were exposed to GST-Ras agarose. The top Western blot contains the GST-Ras precipitant and the lower blot contains the inputs from whole cell lysates with both blots probed with anti-myc antibody. Fig. 6B displays two representative (n=3) Western blots with both containing the HA-immunoprecipitant from cells expressing HA-tagged K-Ras and myc-tagged αENaC together and individually. The top blot was probed with anti-myc antibody and the lower with anti-HA antibody. Both the pull-down experiments and the immunoprecipitation experiments clearly showed an association of ENaC with Ras.

Fig. 7 shows a summary graph of the macroscopic, amiloride-sensitive, inward Na^+ currents at −80 mV in voltage clamped CHO cells expressing hENaC alone, with PI3-K K227E and K-Ras separately and together. The K227E p110α PI3-K mutant has high basal phospholipid kinase activity but is incapable of interacting with K-Ras [23]. This form of active PI3-K failed to augment ENaC activity. Moreover, in the absence of association with K-Ras and presumably ENaC, constitutively active PI3-K (K227E)

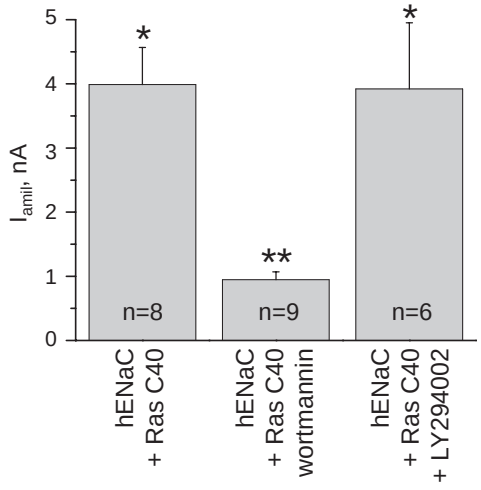

Fig. 5. PI3-K is positioned between K-Ras and ENaC. Summary graph of amiloride-sensitive ENaC currents in cells expressing the channel+PI3-K 1st effector specific mutant Ras (G12:C40) before and after wortmannin and LY294002. *P vs. hENaC alone (Fig. 1), **vs. hENaC+Ras C40, hENaC+Ras C40+LY294002 n.s. vs. hENaC+Ras C40.

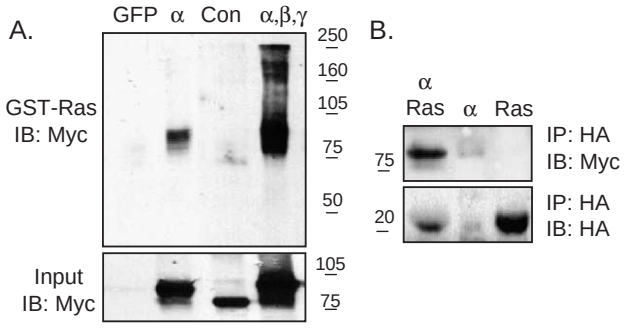

Fig. 6. K-Ras directly interacts with ENaC. (A) Typical Western blots probed with anti-myc antibody containing the precipitant (top) and input (bottom) from GST-Ras pull-down experiments performed on whole cell lysate from cells expressing eGFP, myc-tagged αENaC, a myc-tagged control protein, and myc-tagged α,β and γENaC. (B) Typical Western blots probed with mouse monoclonal anti-myc and anti-Ha antibodies containing the anti-Ha (rat monoclonal) immunoprecipitant from cells expressing HA-tagged K-Ras and myc-tagged αENaC together and individually.

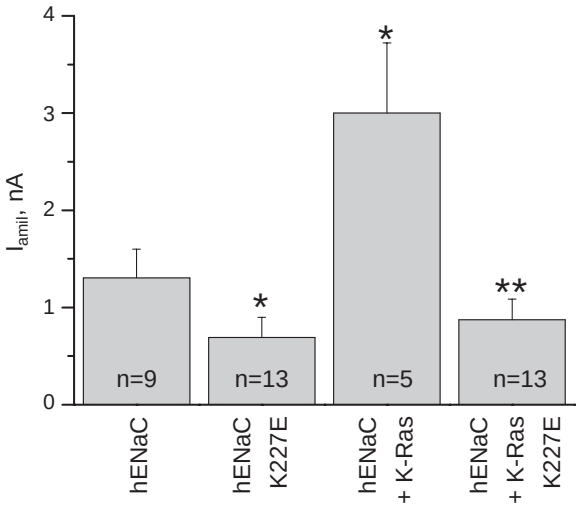

Fig. 7. PI3-K must interact with K-Ras to stimulate ENaC. Summary graph of amiloride-sensitive ENaC currents in cells expressing the channel alone and with the PI3-K K227E mutant, which is incapable of interacting with Ras. Also shown are ENaC activities in the presence of K-Ras alone and with both K-Ras and PI3-K K227E. *P vs. hENaC alone, **vs. hENaC+K-Ras.

functioned in a dominant negative manner even in the presence of overexpressed K-Ras.

We have demonstrated previously that there is a close functional association between PI3-K and ENaC with the phospholipid products of this kinase significantly activating the channel in a membrane delimited manner [15,16]. Fig. 8 shows a representative (n=8) excised, outside-out patch from a CHO cell expressing ENaC containing at least six ENaC. These cells were pretreated with wortmannin (0.2 μM, 5 h) to suppress endogenous PI3-K activity. As shown in the top current trace, the addition of the PI3-K phospholipids product PIP_3 increased ENaC activity. The middle and bottom traces show an expanded time course before (I. control) and after addition of PIP_3 (II). This patch was voltage-clamped at 0 mV with inward Na^+ current being downward. The addition of phospholipid quickly increased ENaC activity from 0.69 to 1.27.

4. Discussion

The current results demonstrate that K-Ras enhances ENaC activity. These findings confirm our previous findings [8] and those of others [5,7,9]. The findings that PI3-K is required for K-Ras actions on ENaC with this phospholipid kinase being a downstream effector of K-Ras positioned between the small G protein and the channel are novel. In addition to stimulating PI3-K to activate ENaC, K-Ras also, in an undetermined manner as yet, associated with ENaC to localize PI3-K near the channel.

The current finding that PI3-K is necessary for K-Ras enhancement of ENaC activity is consistent with previous findings showing that PI3-K is necessary for basal and aldosterone-induced increases in Na^+ reabsorption [10,14,27]. The current findings also fit with the fact that PI3-K is a known 1st effector of K-Ras [19,23] and that aldosterone increases K-Ras levels in epithelial cells through control of

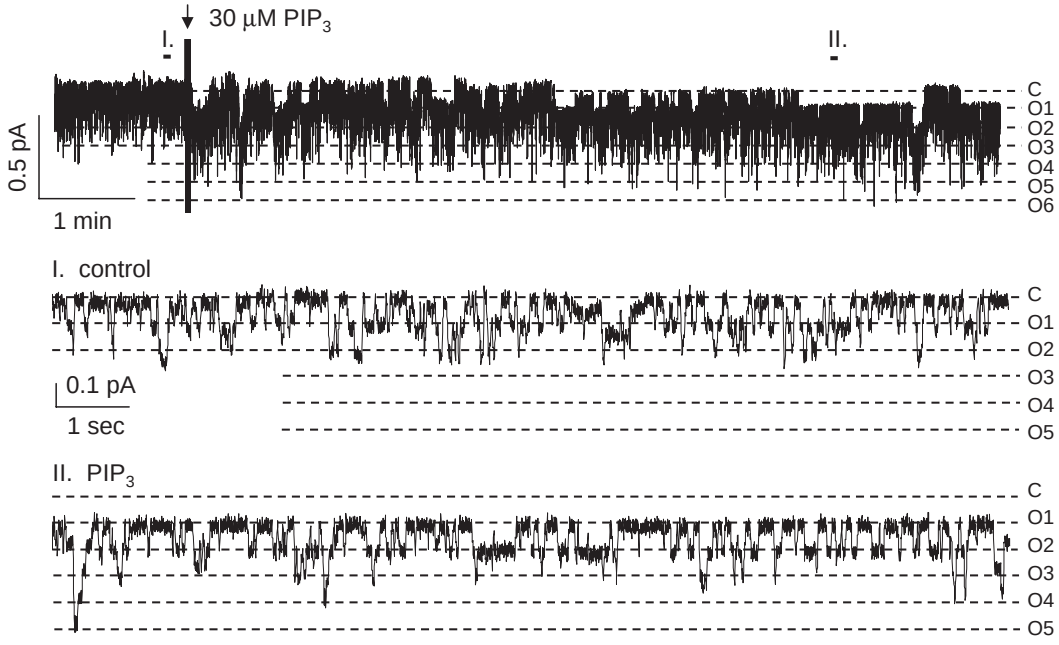

Fig. 8. PI3-K signaling increases ENaC activity. Representative current traces of ENaC in an outside-out patch made from a CHO cell expressing ENaC before and after the application of the PI3-K phospholipid product PIP_3 (30 μM, noted by arrow). Holding potential is 0 mV with inward currents downwards. The top trace shows an extended time period with the middle and lower traces showing segments at expanded scales before (noted with bar labeled I) and after (noted with bar labeled II) the addition of PIP_3, respectively.

transcription of this small G protein [5]. Results from using the 1st effector-specific C40 Ras mutant place PI3-K between the small G protein and the channel. Such a position is consistent with both aldosterone and Ras increasing the levels of the phospholipid products of PI3-K [10,19,23], and with recent findings showing ENaC to be directly activated by the phospholipid products of PI3-K [15,16]. It appears that K-Ras, PI3-K and the phospholipid products of PI3-K all increase ENaC open probability supporting the idea that they are part of a common signaling cascade/mechanism [16,32]. Also consistent with this possibility are the current findings demonstrating that K-Ras and PI3-K are not additive with respect to activating ENaC.

An alternative that cannot be fully excluded with the current data set is that K-Ras and PI3-K are independent with both increasing channel open probability through distinct mechanisms to a maximum value and, thus, show no additive effect. We, however, believe that this alternative unlikely for hydrolysis of GTP is necessary for PI3-K activation of ENaC and a mutant form of Ras (C40), which only interacts and activates PI3-K activated ENaC, whereas, a mutant form of PI3-K with elevated activity but incapable of interacting with K-Ras had no effect on the channel and, indeed, acted in a dominant negative manner with respect to K-Ras augmentation of ENaC activity. The current finding that K-Ras associates with ENaC is novel and exciting. It remains to be determined whether this is a direct or indirect association. We speculate that it is most likely an indirect association for ENaC does not contain recognized domains that would enable direct interaction with this small G protein. We have, however, reported previously that aldosterone promotes the activation and association of PI3-K with K-Ras in epithelia [14]. With this in mind, the present findings suggest that K-Ras has the potential to localize activated PI3-K near the channel. With such a mechanism then the phospholipid products of PI3-K would be well positioned to activate the channel. Further supporting such a function for K-Ras are our results showing that constitutively active (myristoylated) PI3-K capable of localizing to the plasma membrane and interacting with K-Ras augments ENaC activity but that a distinct constitutively active form of PI3-K (K227E), which is unable to interact with Ras [19,23], functions in a dominant negative manner, possibly sequestering necessary PI3-K subunits, such as the regulatory p85 subunit, away from ENaC-associated PI3-K.

We also speculate that association of PI3-K with K-Ras and ENaC protects, in a yet to be determined manner, the phospholipid kinase from competitive inhibitors, such as LY294002, but not irreversible inhibitors, such as wortmannin. While the details of this protection remain to be established, a possibility is that the localization of PI3-K via K-Ras near ENaC places it in a cellular local not directly accessible to competitive inhibitors or leads to the formation of a multi-protein complex that occludes the LY294002 binding site or causes steric changes in PI3-K resulting in a loss of the LY294002 binding site. With any of these possibilities, wortmannin, which could easily covalently modify and, thus, irreversible inactivate PI3-K prior to the latter's association with K-Ras and the channel, would be a functional inhibitor of both K-Ras and PI3-K actions.

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