Abnormal EGF-dependent regulation of sodium absorption in ARPKD collecting duct cells

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Veizis, I. Elias, and Calvin U. Cotton. Abnormal EGF-dependent regulation of sodium absorption in ARPKD collecting duct cells. Am J Physiol Renal Physiol 288: F474–F482, 2005. First published November 2, 2004; doi:10.1152/ajprenal.00227.2004.—Anlioride-sensitive sodium entry, via the epithelial sodium channel (ENaC), is the rate-limiting step for Na⁺ absorption in kidney collecting ducts, and epidermal growth factor (EGF) inhibits Na⁺ transport and ENaC expression. A pathognomonic feature of polycystic kidney disease (PKD) is EGF receptor mislocation to the apical plasma membrane and EGF/EGFR receptor axis overactivity. Immunohistochemical and biochemical analysis revealed mislocation of EGF receptor and excessive activation of the p42/44 extracellular signal-regulated protein kinase pathway (ERK1/2) in kidneys from cystic mice compared with noncystic littersmates. Primary monolayer cultures of noncystic and cystic murine collecting duct principal cells were used to identify aberrant EGF-dependent ERK1/2 activation and regulation of Na⁺ transport associated with autosomal recessive PKD. Addition of EGF to the basolateral bathing solution of noncystic or cystic monolayers led to p42/44 phosphorylation and inhibition of Na⁺ transport (30–35%), whereas apical EGF was effective only in monolayers derived from cystic mice. p42/44 Phosphorylation and inhibition of Na⁺ transport were prevented by prior treatment of the cells with an ERK kinase inhibitor. Chronic treatment (24 h) of noncystic and cystic monolayers with basolateral EGF elicited sustained inhibition of Na⁺ transport (50–55%) and a reduction in steady-state ENaC mRNA levels (50–75%). In contrast, addition of EGF to the apical bathing solution (24 h) had no effect in noncystic monolayers but led to inhibition of Na⁺ transport (50–60%) and decreased ENaC expression (45–60%) in cystic cells. Pretreatment of the monolayers with an ERK kinase inhibitor abolished the chronic effects of EGF on Na⁺ transport. The results of these studies reveal that the mislocalized apical EGF receptors are functionally coupled to the ERK pathway and that abnormal EGF-dependent regulation of ENaC function and expression may contribute to PKD pathophysiology.

mitogen-activated protein kinase; polycystic kidney disease; epidermal growth factor receptor; epithelial sodium channel; autosomal recessive polycystic kidney disease

POLYCYSTIC KIDNEY DISEASES (PKDs) are genetic disorders characterized by formation and progressive enlargement of fluid-filled cysts in the kidney and liver (1, 8). Autosomal dominant (ADPKD) and recessive (ARPKD) forms of the disease ultimately lead to end-stage renal disease and kidney failure in both adults and children. Although the genetics of the PKDs have been defined, the pathogenesis of the disease remains unclear (12). ADPKD cysts originate from all segments of the nephron and frequently disconnect from the tubule (10), whereas ARPKD cysts are more correctly described as dilated collecting ducts (CDs) (13). The most important characteristics of disease development are neoplastic-like increase in cell proliferation (6, 18, 22) and abnormalities in fluid and electrolyte transport (36, 37, 45) that with time fill the cysts and accelerate disease progression. Cell proliferation and altered ion transport may be facilitated by a variety of factors abundantly present in luminal fluid such as growth factors [epidermal growth factor (EGF) and transforming growth factor-α (5, 20), nucleotides (31), and PGE (34) in an autocrine or paracrine fashion and by circulating hormones such as vasopressin (9)].

Cyst epithelial cells in human PKD also exhibit abnormalities in epithelial cell polarity. The EGF receptor (EGFR) localized to basolateral membrane of mature renal tubules is found in both apical and basolateral membranes of cyst epithelial cells (6, 24, 39). Similar mislocalization was observed in several murine models of PKD such as: cpl, orpk, bpk, Pkd1 mutant mice (11), and a recently developed Kif3A knockout model (15). The aberrant localization of EGFR does not reflect a generalized defect in cell polarity because aquaporins-2 and -3 (AQP2 and AQP3) and Na-K-ATPase are localized appropriately (2, 15) in the postnatal kidney. The localization of Na-K-ATPase still remains controversial as some investigators have found immunodetectable levels of the pump at the apical membrane of cystic cells, but in the mouse model of ARPKD used in these studies the Na-K-ATPase is found almost exclusively in the basolateral membrane of noncystic and cystic renal epithelial cells. Furthermore, overactivity of EGF/EGFR axis contributes to PKD pathophysiology, as EGFR inhibition slows disease progression in ARPKD models (27, 40, 42).

Kidney CD is predominantly an absorptive epithelium and electrogenic Na⁺ entry into the principal cells (PC) is mediated by epithelial sodium channel (ENaC) (7, 28). ENaC is composed of three homologous subunits α, β, and γ (3) and channel expression, trafficking, and gating are highly regulated. Although aldosterone is recognized as the major positive regulator of sodium transport in the colon and distal nephron (17, 19, 26), several signaling pathways appear to modulate ENaC-mediated Na⁺ transport in CDs including those activated by growth factors (EGF). In contrast to steroid hormones that increase Na⁺ transport, EGF inhibits CD Na⁺ transport by a poorly defined mechanism(s) (32, 43, 44). Interaction of EGF with its receptor elicits receptor dimerization and phosphorylation, recruitment of accessory proteins, and initiation of several downstream signaling pathways including sequential activation of Ras, Raf-1, Mek-1, and ERK1/2 kinases (30, 41). In parotid salivary epithelial cells, protein kinase C-dependent

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activation of ERK1/2 leads to transcriptional downregulation of the α-ENaC expression (47). Furthermore, it was shown that ERK1/2 phosphorylation antagonized glucocorticoid-dependent activation of α-ENaC gene transcription (16), which is evidence for a cross talk between nuclear receptor and ERK1/2 pathways. Rapid, nongenomic inhibitory effects of EGF on Na⁺ absorption have been reported (43); however, the precise mechanism of ENaC inhibition remains to be defined.

The present study was undertaken to examine the effect of EGF on regulation of Na⁺ transport in CD PC isolated from noncystic and cystic kidneys. Short- and long-term exposure to EGF from either apical (AP) or basolateral (BL) surface was evaluated. The results of these studies demonstrate that acute addition of EGF to the BL bathing solution of noncystic or cystic CD PC stimulates phosphorylation of ERK1/2 and inhibition of amiloride-sensitive Na⁺ transport. In contrast, addition of EGF to the AP bathing solution had no effect on noncystic CD monolayers yet elicited robust activation of ERK1/2 and inhibition of Na⁺ transport in cystic cell monolayers. Long-term exposure to BL EGF was associated with a decrease in Na⁺ transport and steady-state ENaC mRNA expression in noncystic and cystic cells. A similar response was observed in cystic cells but not in noncystic cells when EGF was added to the AP bathing solution. These findings support the concept that mislocalization of EGFRs to the AP membrane of cystic cells allows access to activating ligand present in the luminal fluid and thereby contributes to elevated ERK1/2 phosphorylation in cystic tubules and may be responsible for enhanced cellular proliferation and ion transport abnormalities associated with PKD.

METHODS

CD cell isolation and primary culture. The mice used in this study were obtained by breeding the Hoxb7/EGFP transgenic line (35) with the B6 FVB (Jackson Laboratories, Rockville, MD), supplemented with 5 mg/l insulin, 2.5 mM glutamine, 25 μg/l penicillin G, 1.3 μg/l sodium selenite, 5 μg/l transferrin, 1.3 μg/l triiodothyronine, 50 mM dexamethasone, 30 mM penicillin G, 50 mg/l streptomycin, and 5% FBS (GIBCO-BRL, Gaithersburg, MD) at 37°C. Five to 7 days later, cells were subjected to fluorescence-activated cell sorting (FACS) to collect GFP-positive noncystic (Hoxb7/EGFP-bpk0/H11001) and cystic (Hoxb7/EGFP-bpk0/H11002) CD cell isolation and primary culture.

Immunohistochemistry. Kidneys were dissected from 18-day-old animals (cystic and noncystic GFP-negative littermates) and one kidney was fixed in ice-cold 3.7% paraformaldehyde in PBS and the contralateral kidney was quickly frozen for Western blot analysis. Kidneys were washed for 1 h under running water, dehydrated through serial ethanol and xylene solutions, and embedded in paraffin. Tissue sections (3 μm) were cut from paraffin-embedded kidneys. Immunohistochemistry was performed on deparaffinized, rehydrated sections as previously described (45). Sections were probed with antibodies to EGFR (RDI), E-cadherin (Zymed), and anti-phospho-ERK1/2 (p-ERK1/2; Cell Signaling). Secondary antibodies (from Molecular Probes) were conjugated to either Alexa Fluor 488 or Texas Red. Photomicrographs were obtained with a confocal Zeiss LSM microscope.

To examine EGFR localization in vitro, immunohistochemistry was performed as described (45) on confluent monolayers of cystic and noncystic CD cells grown on collagen-coated permeable supports. Image stacks were acquired with a Zeiss Axioshot microscope equipped with a DG4 light source (Sutter Instrument) and a 12-bit CoolSnapHQ camera (Roper Scientific) under control of Metamorph v4.5 (Universal Imaging). Images were deconvolved by Autoquant’s AutoDeblur software (AutoQuant Imaging).

Immunoprecipitation and Western blot analysis. Kidneys were dissected from noncystic and cystic littermates, capsula was removed, and tissues were washed with PBS and quickly frozen on dry ice. Afterwards, 1 ml of ice-cold RIPA buffer (150 mM Tris, pH 8.0, 150 mM NaCl, 1% IGEPAL, 0.1% Triton X-100, 1 mM sodium orthovanadate, 2.4 mM EDTA, 0.5 mM PMSF) plus protease and phosphatase inhibitor cocktail (according to manufacturer’s instructions, Sigma) was added to the noncystic kidney and 2 ml to cystic kidney. Tissues were homogenized on ice and the lysates were cleared by centrifugation (10 min at 10,000 g). The supernatant was collected and protein concentration was determined using BCA (Pierce, Rockford, IL). EGFR was precipitated from 500 μg of cell lysate protein by 10.220.33.5 on June 23, 2017 http://ajprenal.physiology.org/ Downloaded from by 10.220.33.5 on June 23, 2017 http://ajprenal.physiology.org/ Downloaded from by 10.220.33.5 on June 23, 2017 http://ajprenal.physiology.org/ Downloaded from by 10.220.33.5 on June 23, 2017
portion of the kidney lysate (not used for IPs) was retained for SDS-PAGE and Western blot analysis of MAP kinase signaling components. Cell lysates were also prepared from primary monolayer cultures of CD cells, treated the same as described under electrophysiological studies (acute EGF exposure), for SDS-PAGE analysis of total and phosphorylated ERK1/2. Samples of kidney lysates (20 μg), primary cell culture lysates (10 μg), and protein recovery by immunoprecipitation were boiled in SDS sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue for 10 min. The denatured proteins were separated by either 7.5 or 10% SDS-PAGE. The protein was electrophoretically blotted onto a pure nitrocellulose transfer and immobilization membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked 1 h at room temperature in TBS that contained 5% dry milk (wt/vol), 0.1% polyoxyethyleneisoboritan monolaurate (Tween 20), and 0.01% sodium azide or TBS-3% BSA. After a brief wash to remove the Tween 20, the membranes were incubated overnight at 4°C with specific primary antibodies [anti-EGFR (Research Diagnostics); RC20 anti-p-β (BD Biosciences); anti-p-Raf, anti-p-Mek, anti-p-ERK (BD Biosciences); anti-p-Erk1 (BioSource); anti-p-Elk1 (R&F Signaling)] in TBS-5% dry milk (or TBS-3% BSA for anti-p-β). The membranes were then incubated with secondary antibody (horseradish peroxidase conjugated) at room temperature for 1 h. Membranes were rinsed three times and peroxidase-labeled membranes were developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and protein bands were visualized on X-ray film (X-O-Mat, Kodak, Rochester, NY). Molecular mass estimation of detected bands was determined by using Precision Plus Protein Standards (Bio-Rad). Quantification of the intensity of the bands on the luminograms was determined with Versa Doc Imaging Systems (model 3000, Bio-Rad). The Quantity One (version 4.4.0) density scan program (Bio-Rad) was used to analyze the relevant densities of protein bands.

Quantitative RT-PCR analysis of ENaC mRNA. Cystic and noncystic CD cells were grown on collagen-coated filter inserts and divided in two groups: 1) nontreated (controls) and 2) EGF treatment (20 ng/ml) for 24 h (AP or BL). Total RNA was extracted using the RNeasy Mini Kit, which includes an on-column DNase digestion with RNase-free DNase (Qiagen, Valencia, CA). The concentration and quality of mRNA were determined photometrically (260/280 nm). RNA samples were DNase digested with RNase-free DNase (Qiagen, Valencia, CA). The concentration and quality of mRNA were determined photometrically (260/280 nm). The concentration of mRNA subunit (calculated as a ratio of α-, β-, and γ-ENaC mRNA (copy number) were measured for each EGF-treated sample and the respective control sample. Real-time PCR analysis of all samples (nontreated and EGF-treated cystic; nontreated and EGF-treated noncystic) was performed in a single run for each transcript. Variation in cDNA concentration in different samples was corrected by using the housekeeping gene concentration-GAPDH in each sample. The relative amount of ENaC mRNA subunit (calculated as a ratio of α- or β- or γ-ENaC/GAPDH) present in EGF-treated samples was normalized to the amount of ENaC mRNA subunit (calculated as a ratio of α- or β- or γ-ENaC/GAPDH) in the control samples.

The studies with mice were performed in accordance with the Guide for the Care and Use of Laboratory Animals of National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Case Western Reserve University School of Medicine.

Statistical analysis. All results are expressed as means ± SE and statistical significance was evaluated by either unpaired or paired Student’s t-test. P < 0.05 was considered significant.

RESULTS

EGF and ERK1/2 activation in noncystic and cystic kidneys. EGF/ERK axis overactivity is a feature of cystic tubules in dominant and recessive PKD and disruption of EGF slows disease progression in animal models of ARPKD (39, 42), but the activity of potential downstream signaling pathways such as Ras/Raf/MEK/ERK has not been evaluated. As previously described, EGFR distribution in noncystic renal tubules is primarily at the basolateral membrane and overlaps that of E-cadherin (Fig. 1, A–C). There is basolateral colocalization of EGFR and E-cadherin in cystic tubules, but there is also strong apical staining in these cells (Fig. 1, D–F). The localization patterns of the EGFR in CDs from noncystic and cystic kidneys were retained in polarized primary cultures of CD PC as illustrated in Fig. 7, A and C. The expression levels of EGFR are similar in cystic and noncystic kidney, but the amount of the tyrosine-phosphorylated form of EGFR is significantly elevated in cystic kidneys compared with noncystic (Fig. 1G).

Immunolocalization of p-ERK1/2 in sections of noncystic and cystic kidneys revealed a high level of p-ERK1/2 in epithelial cells lining the cystic tubules with almost no detectable p-ERK1/2 in sections from noncystic kidney (Fig. 2, A and B). Furthermore, Western blot analysis of lysates from noncystic and cystic kidney shows a dramatic increase (6- to 9-fold, n = 16) in the amount of p-ERK1/2 with no difference in total-ERK1/2 (Fig. 2C).

In vivo phosphorylation status of additional components of the EGFR-MAP kinase signaling pathway was evaluated by Western blot analysis of kidneys from three noncystic mice and three cystic littermates. As illustrated in Fig. 3, the phosphorylated forms of MAP kinase kinase (cRaf), MAP kinase kinase (MEK), and MAP kinase (ERK1/2) were significantly increased in cystic compared with noncystic kidneys. Phosphorylation of a downstream transcription factor (Elk1), known to be a substrate of active ERK1/2, was also found to be increased in cystic kidneys. A sustained high level of MAP kinase signaling might be expected to impact cellular proliferation and function in cystic epithelial cells.

Acute effects of EGF on noncystic and cystic CD monolayers. Primary cultures of cells isolated by FACS from Hoxb7/GFP-bpk–/– transgenic mice (noncystic) and Hoxb7/GFP-
bpk+/− (cystic) form polarized epithelial monolayers and express marker proteins and ion transport functions attributable to distal nephron PC. Cultures derived from noncystic mice had mean $I_{sc}$ of 29 ± 4.5 μA/cm² ($n = 17$), due primarily (90%) to amiloride-sensitive sodium absorption. Monolayer cultures derived from cystic mice exhibited 50% lower amiloride-sensitive sodium absorption ($I_{sc} = 14.2 ± 4.8 \mu A/cm^2; n = 18$). The bioelectric properties of normal and cystic cell cultures used in the studies reported herein are similar to those reported previously (45).

Fig. 2. Expression and phosphorylation of ERK in noncystic and cystic mouse kidneys. Sections (3 μm) of paraffin-embedded kidney were prepared and incubated with a primary antibody against p-ERK1/2 (A and B), followed by a secondary antibody conjugated to Texas Red and nuclei were labeled by exposing the sections to DAPI. Images of noncystic and cystic kidney sections were collected and processed in an identical fashion. Note the extensive labeling of p-ERK1/2 in epithelial cell lining the cysts compared with renal tubules from the noncystic mouse kidney. Bar = 40 μm. C: Western blot analysis of total and phosphorylated ERK1/2 from noncystic and cystic kidney (each lane represents a different animal). Kidney lysates were prepared and proteins were separated by SDS-PAGE. Top: samples were probed with an anti-p-ERK1/2 antibody. Bottom: same samples were probed with an anti-total-ERK1/2 antibody.

Fig. 3. In vivo activation of MAP kinase signaling cascade in kidneys from noncystic and cystic BPK mice. Equal amounts of protein (20 μg/lane) isolated from 3 pairs of cystic and noncystic 21-day-old littermates were loaded, electrophoresed, and probed. Immunoblots of p-cRaf, p-MEK1/2, p-ERK1/2, tot-ERK1/2, and p-Elk1 are shown. Each of the active (phosphorylated) components of the MAP kinase signaling cascade is dramatically upregulated in kidneys from cystic mice compared with noncystic littermates.
Addition of EGF (20 ng/ml) to the AP bathing solution of noncystic CD cell monolayers mounted on Ussing chambers had no effect on $I_{sc}$ (Fig. 4A). In contrast, addition of EGF to the BL bathing solution of noncystic CD cell monolayers elicited a monotonic decrease in $I_{sc}$ as illustrated in Fig. 4B. Pretreatment of the monolayer with an ERK kinase inhibitor (30 μM PD-98059, 15 min) completely prevented the EGF-induced inhibition of $I_{sc}$ (Fig. 4C). The data from multiple experiments are summarized in Fig. 6A.

Apical mislocalization of the EGFR is a common feature in PKD, but the coupling to specific signal transduction pathways and the effects of apical EGFR receptor signaling on cellular physiology in cystic cells have not been reported. To determine whether apical EGFRs are functional and can modulate ion transport, monolayers of cystic CD PC were exposed to EGF added to either the AP or BL bathing solution. Similar to what was observed with noncystic CD cell monolayers, addition of EGF to the BL bathing solution reduced amiloride-sensitive $I_{sc}$ by ~35% (Fig. 5C). In contrast to the lack of response of noncystic cells to apical EGF, treatment of cystic monolayers caused inhibition of $I_{sc}$ (Fig. 5A) that was indistinguishable from the response to basolateral EGF (Fig. 6C). Furthermore, the inhibitory effect of apical or basolateral EGF on $I_{sc}$ is completely prevented by pretreatment with an ERK kinase inhibitor (30 μM PD-98059; Fig. 5, B and D). Treatment with PD-98059 has no significant effect on baseline amiloride-sensitive $I_{sc}$ in noncystic or cystic monolayers (Fig. 6, A and B) likely because ERK1/2 phosphorylation is very low under baseline conditions. The ion transport data from multiple experiments are summarized in Fig. 6B. In another set of experiments, EGF was added simultaneously to both apical and basolateral chambers and the inhibitory effects of EGF on $I_{sc}$ were not additive (data not shown). Most likely this is due to full stimulation of ERK1/2 signaling from either apical or basolateral EGF and/or to saturation of a downstream response element. The results presented in the preceding sections showed that the inhibitory effect of EGF on amiloride-sensitive Na$^+$ current was completely abolished by pretreatment with PD-98059, presumably due to a reduction in EGF-induced ERK1/2 phosphorylation and activation. To test this, the effect EGF added to either the AP or BL bathing solution on p42/44 phosphorylation in noncystic and cystic CD cell monolayers was examined. Total and phosphorylated ERK1/2 in lysates from cystic and noncystic cell monolayers treated with vehicle,
EGF (20 ng/ml; AP or BL), and EGF plus PD-98059 (30 μM; AP and BL) was measured. Whereas total ERK1/2 did not vary between the conditions, basolateral exposure to EGF dramatically increased the phosphorylation of ERK1/2 in both non-cystic and cystic cell monolayers (Fig. 7, B and D). In contrast, addition of EGF to the AP side of noncystic CD cell monolayers did not increase ERK1/2 phosphorylation, but treatment of cystic CD cell monolayers elicited a robust phosphorylation of ERK1/2. Pretreatment with PD-98059 completely blocked EGF-induced ERK1/2 phosphorylation (Fig. 7, B and D).

Modulation of electrogenic sodium transport by long-term exposure to EGF. Previous work from our laboratory demonstrated that prolonged exposure of mCT1 cells (mouse CD cell line) to EGF inhibits amiloride-sensitive Na+ absorption (32) due to a reduction in ENaC-mediated apical Na+ entry. The effects of chronic exposure to EGF in noncystic and cystic primary CD cell monolayers were determined. Confluent epithelial monolayers of noncystic or cystic cells were exposed to either apical or basolateral EGF (20 ng/ml) for 24 h and amiloride-sensitive $I_{sc}$ was measured. As summarized in Fig. 8A, addition of EGF to the apical side of noncystic monolayers had no effect on $I_{sc}$, whereas basolateral EGF reduced $I_{sc}$ by $\sim 50\%$ (28.7 ± 3.2 to 15 ± 2.6 μA/cm², $n = 4$). Chronic exposure of cystic cell monolayers to EGF from either the apical or basolateral surface significantly inhibited $I_{sc}$ (Fig. 8B) by 60–65% (control 16.7 ± 2.4, $n = 4$; apical EGF 8.2 ± 1.6, $n = 5$; basolateral EGF 6.7 ± 2.1 μA/cm², $n = 4$). The inhibitory effects of EGF on amiloride-sensitive $I_{sc}$ were not observed in monolayers pretreated with the ERK1/2 kinase inhibitor PD-98059.

Effects of long-term EGF treatment on α-, β-, and γ-ENaC mRNAs. The acute effects of EGF on $I_{sc}$ probably reflect a change in channel gating or trafficking, whereas the sustained reduction of amiloride-sensitive $I_{sc}$ with prolonged exposure to EGF may involve genomic regulation. To investigate the mechanism of long-term (24 h) EGF-dependent, ERK1/2-mediated ENaC regulation of sodium transport, steady-state mRNA levels of all three ENaC subunits were measured in primary monolayer cultures of noncystic and cystic CD PC. Real-time RT-PCR was used to quantify the mRNAs for the three ENaC subunits and for GAPDH. For each treatment
EGF REGULATION OF SODIUM ABSORPTION

Fig. 8. Effects of long-term treatment of noncystic and cystic CD cells with EGF on amiloride-sensitive sodium absorption. Confluent monolayers of CD cells isolated from noncystic and cystic mice were treated for 24 h with either vehicle, EGF (20 ng/ml, apical or basolateral), or PD-98059 (30 μM, apical and basolateral) plus EGF. The cultures were then transferred to Ussing chambers and after 20 min, amiloride was added to the AP bathing solution to inhibit amiloride-sensitive \( I_{\text{sc}} \). A: monolayer cultures derived from noncystic mice \((n = 4 \text{ for each condition})\). B: monolayer cultures derived from cystic mice \((n = 3–5 \text{ for each condition})\). *Significantly different from vehicle-treated cultures \((P < 0.05, \text{unpaired } t\text{-test})\).

Fig. 9. Effects of long-term treatment of noncystic and cystic CD cells with EGF on steady-state mRNA levels for epithelial sodium channel (ENaC) subunits. Confluent monolayers of CD cells isolated from noncystic \((A)\) and cystic \((B)\) mice were treated for 24 h with vehicle or EGF \((20 \text{ ng/ml, apical or basolateral addition})\). Total RNA was harvested and the samples were reverse transcribed. Each sample was analyzed for GAPDH, α-ENaC, β-ENaC, and γ-ENaC mRNAs. Standard curves for each of the cDNAs were constructed and copy number was determined. The ratio of each channel subunit to GAPDH for vehicle-treated samples was calculated and assigned a value of 100%. The corresponding ratios for EGF-treated samples were determined and expressed as percent of the value in the absence of EGF. The data represent means ± SE for 3–4 independent experiments. *Significantly different from vehicle-treated cultures \((P < 0.05, \text{paired } t\text{-test})\).

(EGF) and control sample. GAPDH mRNA and α-, β-, γ-ENaC mRNA were measured, and the data are normalized to GAPDH and expressed as a percentage of control (no treatment). The steady-state mRNA levels for GAPDH were not changed by EGF treatment of the monolayers [the mean values for GAPDH mRNA of treated noncystic cultures were 103 ± 5% \((n = 4)\) and cystic 104 ± 4% \((n = 6)\)]. The results are summarized in Fig. 9. Chronic exposure of primary CD cells to EGF from the basolateral side \((20 \text{ ng/ml, } 24 \text{ h})\) decreased the abundance of all three ENaC subunits in noncystic \((\alpha, \beta, \text{ and } \gamma; \text{Fig. 9A})\) and cystic cells \((\alpha, \beta, \text{ and } \gamma; \text{Fig. 9B})\). In contrast, exposure to EGF from the apical side caused downregulation of ENaC mRNA in cystic \((\alpha, \beta, \text{ and } \gamma; \text{Fig. 9B})\) but not in noncystic CD cell cultures (Fig. 9A).

DISCUSSION

The primary objective of this study was to determine the impact of mislocalized EGFRs on MAP kinase signaling and regulation of sodium absorption in primary cultures of normal and ARPKD CD cells. Previous studies demonstrated that genetic or pharmacological modulation of EGFR activity results in attenuation or slowing of the development of renal disease \((27, 40, 42)\). Mislocalized apical receptors can bind EGF, autophosphorylate, and are mitogenic \((38)\) but the signaling pathway(s) downstream from the mislocalized apical receptors have not been delineated. Although our studies do not demonstrate directly that EGFR signaling is responsible for in vivo activation of MAP kinase cascade in cystic kidneys, it is a likely mechanism as ERK1/2 phosphorylation was prominent in cystic tubules known to express apical EGFRs \((\text{Figs. 1 and 2})\). Enhanced in vivo phosphorylation of ERK1/2 in cystic kidneys \((\text{Figs. 2 and 3})\) suggests a role for MAP kinase signaling in cell growth and function. Activation of basolateral EGFRs in perfused rabbit CDs \((21)\) elicited rapid inhibition of sodium absorption and chronic treatment of a mouse CD cell line with EGF caused ERK1/2-dependent inhibition of sodium transport \((32)\). It is clear that both noncystic and cystic cells respond to exogenous EGF added to the basolateral surface with robust phosphorylation of ERK1/2 \((\text{Fig. 7})\) and acute inhibition of amiloride-sensitive sodium absorption \((\text{Fig. 6})\). Perhaps more importantly, mislocalization of EGFRs to the apical membrane in cystic cells results in acquisition of sensitivity to EGF added to the AP bathing solution \((\text{Figs. 5 and 6})\). Phosphorylation of ERK1/2 and inhibition of \( I_{\text{sc}} \) by EGF are prevented by pretreatment with an ERK kinase inhibitor; therefore, MAP kinase signaling is at least necessary for acute inhibition of sodium transport by EGF. Because primary cultures of cystic CD cells do not exhibit substantially enhanced ERK1/2 phosphorylation in the absence of exogenous ligand, the dramatically higher level of phosphorylation observed in vivo \((\text{Figs. 2 and 3})\) is probably due to enhanced ligand availability and/or aberrant expression of apical EGFRs that
can be activated by ligand constitutively present in the luminal fluid. Alteration in ligand processing, increase in EGFR-independent signaling, or decrease in phosphatase activity could contribute to enhanced MAP kinase signaling in cystic kidneys. The finding that prolonged (24 h) exposure of cultured CD cells to EGF causes a significant reduction in amiloride-sensitive sodium transport and expression of ENaC subunits is consistent with ERK1/2-dependent changes in gene expression in CD PC. Similar effects would be predicted in vivo as: 1) EGFRs are mislocalized to that apical membrane in cystic CDs, 2) EGF and EGF-like molecules are present in urine, 3) there is overactivity of EGF/EGFR axis, 4) phospho-ERK1/2 is elevated in cystic CD cells, and 5) MAP kinase cascade signaling is substantially increased in cystic kidneys. Thus abnormal regulation of amiloride-sensitive sodium transport by apically localized EGFRs seems to be a feature of ARPKD.

The molecular mechanisms responsible for acute and chronic inhibition of sodium transport by EGF are unknown. Because both the acute and chronic inhibitory effects of EGF are prevented by pretreatment with an ERK kinase inhibitor, ERK1/2 phosphorylation is required for EGF-dependent regulation of ENaC activity. The characteristic rapid stimulatory effect of EGF on ERK1/2 phosphorylation and resultant inhibition of amiloride-sensitive \( I_{ac} \) suggests a change in ENaC gating (i.e., decrease in channel open probability, perhaps due to phosphorylation of the channel or a regulatory protein) or an increase in endocytosis and decrease of the number of active channels present in apical membrane. Indeed, ERK1/2-dependent phosphorylation of ENaC near the PY motif present in the COOH terminus of the \( \beta- \) and \( \gamma- \) subunits (33) has been suggested to increase its affinity for a ubiquitin ligase implicated in channel retrieval from the plasma membrane (4, 29).

The inhibitory effect of long-term exposure to EGF is probably mediated by transcriptional downregulation or a decrease in mRNA stability as EGF leads to a decrease in steady-state mRNA levels for all three ENaC subunits. Sustained ERK1/2 activity may interfere with nuclear receptor function (glucocorticoid or mineralocorticoid receptor) by either direct phosphorylation of nuclear receptors (14) or by activation of a repressor of the ENaC transcription complex (46). Additional studies will be required to define the precise mechanisms of acute and chronic regulation of ENaC activity by EGF.

We reported previously that primary cultures of CD PC isolated from normal and BPK mice differ primarily in the magnitude of amiloride-sensitive sodium absorption, with \( \sim 50\% \) reduction in cystic monolayers. The molecular basis for the lower rate of amiloride-sensitive sodium transport in the cystic cells is not known. However, synthesis of EGF or EGF-like ligands may be increased in primary cultures of cystic cells or cystic cells may be more sensitive to autocrine/paracrine regulation by released ligands, but there is not an obvious increase in ERK1/2 phosphorylation in cystic cell monolayers under basal in vitro conditions (Fig. 7).

Many of the epithelial cysts present in advanced ADPKD lack upstream connections and fluid accumulation must be driven by net secretion of salt and water (36, 37). Because ADPKD is a slowly progressing disease and cysts are thought to form at multiple sites along the nephron, it is difficult to ascertain with certainty the segment of origin of a particular cyst and document changes in ion transport that accompany cyst development. In contrast, ARPKD progresses rapidly, and the cysts are derived primarily from collecting tubules and actually represent dilated nephrons with upstream and downstream connections rather than anatomically isolated cysts. Net fluid secretion is not required for cyst enlargement, as an increase in secretion or a decrease in absorption would favor retention of luminal fluid and enlargement of cystic tubules in face of destruction of kidney architecture and pseudodobstruction. We recently reported that primary cultures of noncystic and cystic CD PC maintained under basal conditions (i.e., without exogenous EGF) had similar \( Cl^- \) secretory responses to elevation of \( cAMP \) or calcium (45). Elimination of a \( cAMP \)-regulated \( Cl^- \) channel, implicated in ADPKD fluid secretion, did not affect renal cystic disease in a murine model of ARPKD (23). Non-CFTR \( Cl^- \) channels could make a significant contribution to in vivo fluid secretion that is not apparent when cells are placed in primary culture (e.g., hyperactivity of the EGF/EGFR axis and enhanced ERK1/2 activation may promote fluid secretion). These observations suggest that abnormal regulation of sodium reabsorption, perhaps in combination with enhanced \( Cl^- \) secretion, would be expected to contribute to luminal fluid retention and tubule dilatation in ARPKD.

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