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Epidermal growth factor-mediated proliferation and sodium transport in normal and PKD epithelial cells

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Abstract

Members of the epidermal growth factor (EGF)-family bind to ErbB (EGFR)-family receptors which play an important role in the regulation of various fundamental cell processes including cell proliferation and differentiation. The normal rodent kidney has been shown to express at least three members of the ErbB receptor family and is a major site of EGF ligand synthesis. Polycystic kidney disease (PKD) is a group of diseases caused by mutations in single genes and is characterized by enlarged kidneys due to the formation of multiple cysts in both kidneys. Tubule cells proliferate, causing segmental dilation, in association with the abnormal deposition of several proteins. One of the first abnormalities described in cell biological studies of PKD pathogenesis was the abnormal mislocalization of the EGFR in cyst lining epithelial cells. The kidney collecting duct (CD) is predominantly an absorptive epithelium where electrogenic Na^+ entry is mediated by the epithelial Na^+ channel (ENaC). ENaC-mediated sodium absorption represents an important ion transport pathway in the CD that might be involved in the development of PKD. A role for EGF in the regulation of ENaC-mediated sodium absorption has been proposed. However, several investigations have reported contradictory results indicating opposite effects of EGF and its related factors on ENaC activity and sodium transport. Recent advances in understanding how proteins in the EGF-family regulate the proliferation and sodium transport in normal and PKD epithelial cells are discussed here.

Keywords

Polycystic kidney disease; ADPKD; ARPKD; ENaC; epidermal growth factor; ErbB receptor; ErbB2

1. Introduction

Polycystic kidney disease (PKD) is a common genetic disease that is associated with a high morbidity and mortality [1–6]. Autosomal dominant (ADPKD) and autosomal recessive

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(ARPKD) polycystic kidney diseases are characterized by the progressive growth and expansion of cysts or dilatation of collecting ducts (CD), respectively, that ultimately destroy the normal renal parenchyma. ADPKD is one of the most common monogenic diseases and in 50% of cases results in end-stage renal disease (ESRD) [4,7]. ARPKD is a rare genetic disorder and typically presents in utero or during the neonatal period. ADPKD is caused by mutation in *PKD1* or *PKD2* genes. Mutation in the *PKHD1* gene is responsible for ARPKD [4,8]. At first glance, it seems that the three proteins polycystin-1 (PC-1), polycystin-2 (PC-2) (encoded by *PKD1* or *PKD2*, respectively), and fibrocystin (encoded by *PKHD1*) are involved in cyst formation and might be regulated by similar mechanisms. There are, however, important differences that suggest that regulation of ADPKD and ARPKD are more complicated than anticipated [9]. In ADPKD, cysts, which can arise from any tubular segment, sprout from the nephron and no longer communicate with the tubule from which they originate. The majority of cysts disconnected from tubular structures still accumulate fluid within the lumen [6,10]. In contrast, cysts in ARPKD are dilated CDs that remain contiguous with the remaining nephron, allowing urine to continue to flow through the collecting system [6,11].

The CD is a final site of renal regulation of Na^+ and water balance. Within the CD, Na^+ diffuses from the lumen into principal cells through the epithelial Na^+ channel (ENaC) and is extruded at the basolateral membrane in exchange for uptake of K^+ by the Na/K-ATPase. Cellular proliferation and fluid secretion can be accelerated by growth factors such as epidermal growth factor (EGF). EGF is a small mitogenic protein that is thought to be involved in mechanisms such as normal cell growth. ErbB receptors have been recognized as targets in anticancer therapy and are now used in the treatment of breast and colon malignancies. Other than tumor biology, EGF signaling is critically involved in renal electrolyte homeostasis [12]. Moreover, EGF plays an important role in the expansion of renal cysts. Cyst growth is the result of proliferation of incompletely differentiated epithelial cells and accumulation of fluid within the cysts. Epithelial cells from cysts from patients with both ADPKD and ARPKD are unusually susceptible to the proliferative stimulus of EGF [6]. Moreover, cyst fluids from these patients contain mitogenic concentrations of EGF, and this EGF is secreted into the lumens of cysts in amounts that can induce cell proliferation [13]. It was first shown that EGF is an important mediator of the proliferative abnormality seen in cyst formation in human ADPKD and that this involves a mislocalization of the EGFR to the apical epithelial cell membranes [13]. Subsequent studies found this to be a common feature of PKD, being observed not only in human ADPKD and ARPKD, but also in a variety of spontaneously occurring mouse models of ARPKD, as well as mouse models derived by targeted mutations of genes responsible for ADPKD. Here we discuss recent results supporting functional interactions between EGF-family proteins, proliferation and sodium transport in normal and PKD cells, as well as detailing the molecular mechanisms underpinning such interactions and discuss the biological consequences of these regulations. Hence we aim to provide critical insight into the physiological control of cell proliferation and ion channel function by EGF and its related growth factors.

2. ErbB family

The epidermal growth factor receptor (EGFR) family, also known as ErbB receptors, consists of four transmembrane receptors belonging to the receptor tyrosine kinase (RTK) superfamily and includes EGFR (ErbB1/HER-1), ErbB2 (neu/HER-2), ErbB3 (HER-3), and ErbB4 (HER-4) [14]. The gene symbol for EGFRs, *ErbB*, originates from the name of a viral oncogene to which these receptors are homologous: Erythroblastic Leukemia Viral Oncogene. EGFR was the first discovered RTK [15]. Since then, EGFR (ErbB1/HER-1) is the best characterized and best known ErbB receptor. Most of the principles and paradigms

that underlie the action of RTK were first established for the EGFR [14,16]. In the following discussion, we use the term "ErbB receptors" for the whole ErbB-family and the term "EGFR" in description of experiments where only ErbB1 (EGFR/Her-1) is examined.

EGFR as well as other ErbB receptors is a transmembrane receptor consisting of an extracellular ligand-binding domain with conserved two cysteine-rich domains necessary for ligand binding; a single membrane-spanning domain which has a passive role in signaling and functions as an "anchor" of receptor in plasma membrane [17] and a cytoplasmic protein tyrosine kinase domain, where six tyrosine autophosphorylation sites are located. After ligand binding, ErbB receptors dimerize, which is a required critical step for intrinsic RTK to be activated and specific tyrosine-containing residues become autophosphorylated [18]. Phosphorylated ErbB receptors can recruit different adapter proteins with Src homology domain-2 (SH2) or phosphotyrosine binding domains (PTB) [19]. Signals from dimerized, activated ErbB receptors lead to activation of multiple intracellular signal transduction pathways including cell growth, proliferation, survival/decreased apoptosis, migration etc. Depending on the pairing of ErbB family receptors (homodimers or heterodimers) there can be downstream stimulation of different combinations of signaling pathways [20].

Although EGFR is the predominant ErbB receptor expressed in the normal adult mammalian kidney tubule, ErbB2 and ErbB4 have also been detected in developing ureteric buds [21,22]. However, major roles for ErbB2, ErbB3 and ErbB4 in neural and cardiac development are demonstrated in null mice by embryonic lethality at embryonic day (E)10 prior to the onset of metanephric development [21]. We have shown recently that all ErbB receptors are expressed at least at low levels in cultured principal mpkCCD$_{c14}$ cells. However, ErbB2 is the most predominant member of the ErbB family in these cells [23]. Both ErbB2 and ErbB4 have been detected in adult human and rat CDs, respectively [24]. We have recently also detected ErbB2 in adult rat CDs. Fig. 1 shows representative immunohistochemistry staining for ErbB2 at 40× and 100× magnifications in a cortical section of Sprague-Dawley (SD) rat kidneys. Immunohistochemistry analysis was performed as described previously [23,25]. A negative control (left) (stained with secondary antibodies in the absence of primary antibodies) is also shown. Additional controls (without primary and secondary antibodies) were also negative (data not shown). As clearly seen in Fig. 1, ErbB2 is highly expressed in the CD of SD rat kidney and is localized mostly at the basolateral surface.

ErbB receptors are predominantly located at the basolateral surface of normal adult polarized tubule epithelial cells, but on the apical surfaces of fetal ureteric bud-derived collecting ducts [187,188,191]. ErbB ligands have autocrine properties, which may explain preferential co-localisation of the ligands and their receptor at the basolateral surface. Development of epithelial cell polarity requires a well orchestrated, domain-specific segregation of lipids, membrane proteins, and cytoskeleton. Intracellular transport to the plasma membrane is regulated in part by intrinsic signals recognized at different steps in polar sorting pathways. A number of polar sorting determinants have now been identified in membrane proteins, and principles for both sequence and structural requirements are beginning to emerge. For example, a 23-amino acid segment located near the cytoplasmic face of the membrane spanning domain (residues Lys-652 to Ala-674) is necessary and sufficient for targeting EGFR from the *trans*-Golgi network directly to the basolateral plasma membrane [26,27]. ErbB receptor localization and regulation will be discussed further below.

3. EGF and its related growth factors

Regulation of ErbB receptor function is controlled by their ligands, members of the EGF-related peptide growth factor family [28]. All EGF-family members, like many other growth factors, are derived from membrane-bound precursor proteins [29,30]. There are over 12 ligands that can bind ErbBs and induce dimerization of distinct functional receptor pairs. EGF, transforming growth factor-α (TGFα), and amphiregulin are specific for EGFR and primarily induce the formation of EGFR/EGFR homodimers and EGFR/ErbB2 heterodimers [31]. Betacellulin, heparin binding EGF (HB-EGF), and epiregulin can bind to either EGFR or ErbB4. Neuregulins-1 and -2 can bind both ErbB3 and ErbB4; neuregulins-3 and -4 are specific only for ErbB4. Currently, no known ligands have been identified for ErbB2 homodimers. However, ErbB2 is the preferred heterodimerization partner of other ErbB family members and ErbB2-containing heterodimers have the strongest signaling output [32,33]. The sequence of the ErbB3 catalytic domain suggests that this receptor does not have RTK activity [34]. Thus ErbB3 may function as a platform for heterodimerization and subsequent transphosphorylation by other members of the ErbB family. Hence ErbB2 and ErbB3 can be activated through heterodimerization [34,35]. It was proposed recently that the kinase domain of ErbB3 retains phosphoryl-transferase activity. Kinase activity appears to be ~1,000 fold weaker than for EGFR but may be sufficient for receptor *trans*-phosphorylation in the context of an ErbB heterodimer [36].

ErbB ligands exist as inactive transmembrane precursors, and need the proteolytic cleavage of their ectodomain to be released as mature soluble ligands. All EGF-family members are synthesized as membrane-anchored precursors that can be processed by specific metalloproteases to release soluble bioactive factors from the cell surface. This cleavage is performed by ADAM (a disintegrin and metalloprotease) family members [37–40] and tightly regulated by different factors. For example, intravenous Angiotensin II (Ang II) infusion induced ADAM17 redistribution to the apical cell membrane, followed by release of TGF-α and activation of the EGFR [41]. This led to the development of glomerulosclerosis, interstitial infiltration of monocytes, fibrosis, and proteinuria. ErbB receptor ligands are cleaved by multiple ADAMs, including ADAM9, 10, 12, 15, 17, and 19. In addition to these ErbB receptor ligands, many ADAMs have multiple substrates, and thereby appear to be involved in various signaling pathways and cellular functions [40]. A role for matrix metalloproteinases (MMPs) in cleaving of ErbB receptor ligands is also proposed [42,43]. MMPs are a large family of zinc-contained proteolytic enzymes that have the capability to degrade extracellular matrix proteins [44]. It was shown recently that MMP9 protects mesenchymal cells from apoptosis during kidney development and stimulates ureteric bud branching morphogenesis, most likely by releasing the soluble form of stem cell factor, suggesting that normal renal development requires MMP9 [45].

4. Activation of ErbB receptors and physiological functions

Addition of ErbB receptor ligands to tubular cells promotes several biological responses, including cell proliferation. The physiological role of ErbB receptors and particularly of EGFR and ErbB2 is well established, especially in cancer. Activating mutations and overexpression of members of the ErbB-family have been implicated in a variety of cancers, including mammary and squamous carcinomas, glioblastomas, lung cancer as well as other malignant diseases [14,46,47]. The role of ErbB-family members in renal development, physiology and pathophysiology is also well recognized [21]. Under physiological conditions, ErbB activation appears to play an important role in the regulation of renal hemodynamics and electrolyte handling by the kidney, while in different pathophysiological states, ErbB activation may mediate either beneficial or detrimental effects to the kidney [21]. ErbB signaling is critically involved in cell signaling, cell growth, proliferation and

renal electrolyte homeostasis. Most of the ErbB-family ligands are expressed in the kidney. For example, HB-EGF is synthesized in the proximal tubule, EGF in thick ascending limb of Henle's loop (TAL) and distal convoluted tubule (DCT), and TGFα in the DCT and collecting ducts [48,49]. However, EGF and its related growth factors are present in the lumen at levels several orders higher than in plasma. As described above, ErbB receptors are expressed at the basolateral surfaces of normal adult kidneys. Thus, ErbB receptors are not accessible to their ligands in the lumen. This may serve as a protective mechanism for the distal nephron. One of such examples is mislocalization of ErbB receptors in PKD which will be discussed below. Another potential mechanism is a disturbance of epithelial cellular integrity. Following tubular injury, tight junctions can be disrupted and thus allow ErbB receptor ligands to traverse to the basolateral surface and consequently activate corresponding receptors. It was shown that EGF via the Raf-1/ERK1/2 pathway regulates the content and distribution of claudins [50,51], proteins that are the most important components of the tight junctions, where they establish the paracellular barrier that controls the flow of molecules in the intercellular space between epithelial cells [52]. HB-EGF has also been shown to play an important role in the regulation of tight junction proteins and transepithelial resistance [53,54].

As discussed above, activation of ErbB receptors is accomplished through ligand binding to the extracellular domain, followed by receptor homo- or heterodimerization and tyrosine transphosphorylation. Combinatorial interactions are very important in ErbB receptor polarization and downstream signaling since ErbB homodimers and heterodimers differ in their biological properties [55].

Once activated by site-specific phosphorylation, ErbB receptors serve as molecular integrators through either direct phosphorylation of target molecules or by serving as scaffolds for adaptor proteins. Activation of ErbB receptors leads to initiation of various signaling cascades. The great diversity of ligands and receptor dimer pairs allows for the activation of numerous signaling pathways that coordinately regulate complex processes including developmental growth control and adult homeostasis. Fig. 2 demonstrates one of such examples when ErbB receptor is phosphorylated and misslocalized during pathophysiological conditions. This figure shows immunohistochemistry staining for phosphorylated ErbB2 (pErbB2) at 40× in a cortical section of SD rat kidneys and in PCK rats (Charles River Laboratories International, Inc., Wilmington, MA) at 40× and 100× magnifications. The PCK rat harbours an autosomal recessive *Pkhd1* gene mutation and is a model of ARPKD [56]. As clear seen in this figure, pErbB2 is upregulated in cystic kidneys compared to normal rat kidney and is localized at the apical membrane of CDs (Fig. 2).

5. PKD

Polycystic kidney diseases (PKD) are a group of inherited disorders characterized by development of renal cysts. Of these, the most common lethal monogenic forms are ADPKD, characterized by progressive development of fluid-filled cysts; and the mainly infantile, ARPKD [4,6,11,57]. ADPKD is inherited as a dominant trait, initiated in utero but usually leading to onset of renal failure in mid-life. It is characterized by early separation of focal spherical cysts from all segments of the nephron and progressive expansion by cell proliferation and fluid secretion throughout life. In contrast, ARPKD is inherited as a recessive trait, often lethal in the perinatal period, and is characterized by ecstatic distension of CDs to form numerous spindle-shaped renal cysts [4–6,11,58]. Because of these differences, ADPKD and ARPKD are treated as two distinct diseases in clinical practice [59].

5.1. ADPKD

ADPKD is one of the most common mono-genetic diseases affecting humans [60] and occurs approximately in 1 in 800 live births. Recent advances in ADPKD are thoroughly reviewed by Torres and Harris [5]. The primary phenotype of ADPKD is the progressive development in both kidneys of multiple fluid filled cysts, which eventually result in ESRD [6]. Two genes have been shown definitively to result in ADPKD when mutated: *PKD1*, which accounts for 85%, and *PKD2*, which is responsible for 15% [3]. The *PKD1*- encoded protein, polycystin-1 (PC-1) is a large (~460 kDa) membrane protein with a long, modular extracellular N-terminal component capable of protein-protein interactions, 11 transmembrane domains, and a short (~200 amino acid) intracellular C-terminal domain [61] which is capable of physical interactions with the *PKD2*-encoded polycystin-2 (PC-2), proteins of focal adhesion and cell-cell adherent junctional complexes as well as the α1-subunit of NaK-ATPase [62,63]. mRNA and protein expression studies have shown that PC-1 is developmentally regulated and highly expressed in fetal kidneys in ureteric bud-derived CD [64–66]. On the basis of its predicted structure and functional features, PC-1 is postulated to be a plasma membrane receptor involved in cell–cell interaction and the regulation of several signaling pathways linked to cell proliferation [6,67–69]. The PC-1 C-terminal domain also contains specific tyrosine (Y) and serine (S) sites for phosphorylation. PC-1 and PC-2 interact physically [62,65,70–73] and form a Ca^{2+}-permeable non-selective cation channel when expressed heterologously [63,74,75]. Furthermore, it was recently shown that PC-1/PC-2 complex regulates pressure sensing [76]. A newly identified coiled-coil domain located in the C-terminus of PC-2 has been shown to mediate dimerization of the C-terminus of PC-2 and recognition of PC-1 [77] and is proposed to be essential for assembly and function of heteromeric polycystin complexes [78].

PC-2, also called TRPP2, belongs to the transient receptor potential (TRP) superfamily of ion channels [79–81]. PC-2 has six transmembrane domains, a pore forming region, and cytoplasmic N- and C-terminal tails [81–83]. In addition to forming a heteromeric complex with PC-1, PC2 has also been proposed to form heteromeric channels with TRPV4 and TRPC1 [84–86]. It was shown that PC-2 and TRPV4 form a mechanical and thermal sensor in cilia and suggested that TRPV4 is the mechanically sensitive component of the complex [84]. The PC-2/TRPC1 heteromeric channel is activated in response to GPCR activation and shows a pattern of single-channel conductance, amiloride sensitivity and ion permeability distinct from that of PC-2 or TRPC1 alone [85]. Kobori et al. using atomic force microscopy recently proposed that PC-2 and TRPC1 form a hetero-tetramer with a 2:2 stoichiometry and an alternating subunit arrangement [86]. Several protein-protein interactions have been reported previously between TRP channels, most of them are known to occur between members of the same group. Similarly to PC-2/TRPC1 and PC2/TRPV4 complexes, we have shown recently that members of other subfamilies of TRP channels, TRPA1 and TRPV1, can assemble into a complex on the plasma membrane and that TRPV1 influences intrinsic characteristics of the TRPA1 channel [87].

5.2. ARPKD

ARPKD is a hereditary renal cystic disease in infants and children affecting the kidneys and liver and occurs in approximately 1 in 20,000 live births. Renal failure and hepatic fibrosis develop in most babies who survive the perinatal period. The disease is characterized by the expansion and elongation of collecting tubules into multiple small cysts [6]. ARPKD is caused by mutations in the *PKHD1* (polycystic kidney and hepatic disease 1) gene [88–90]. The *PKHD1*-encoded protein, fibrocystin-1 (polyductin) spans the cell membrane of kidney cells and is developmentally regulated. Based on fibrocystin domain structure, intracellular phosphorylation sites and capability to physically interact with PC-2, it is thought to act as a mechano-receptor, receiving external signals and initiating various intracellular signaling

processes. This protein also may be involved in adhesion and proliferation. However, exact role of fibrocystin is unknown. Although the human genome contains a *PKHD1* homolog, *PKHDL1*, this is not ARPKD-associated but is thought to be an ancestral variant of *PKHD1* [91]. Fibrocystin-1 has been shown to be associated with the basal bodies/primary cilia of epithelial cells [92–94] and co-localized with PC2 *in vitro* and *in vivo* [95–97]. We have shown recently that PKHD1 mutation in human ARPKD is associated with alterations in cell spreading, matrix adhesion, and migration due to abnormalities of focal adhesion protein (FAK, c-Src, paxillin) content, distribution and activation by site-specific phosphorylation [98].

The overexpression and abnormally located ErbB receptors on the apical surface of cyst-lining epithelia in human and murine models of ARPKD have been shown specifically to bind with high affinity EGF/TGF-α. Overexpressed ErfB receptors autophosphorylate and generate mitogenic signals in response to growth factors [99–102]. However, surprisingly, neither overexpression nor mislocalization of EGFR was seen in PCK rats, the only current genetically orthologous rodent model of ARPKD. Moreover, the specific inhibitors of EGFR(ErbB1): EKI-785 and EKB-569, had no effect on renal cystic progression or fibrocystic liver disease [103]. Subsequently, high expression levels of ErbB2 were observed in the renal cystic epithelia in PCK rats [104]. More recently we have not only observed that ErbB2 is predominantly localized on the basolateral surface in the PCK rats (data not shown) but also shown that phosphorylated pErbB2 is abnormally expressed and mislocalized on the apical surface of cystic epithelia (Fig. 2) compared to normal rat CDs.

6. EGF-mediated proliferation

EGF-ErbB receptor-mediated interactions are key elements in renal tubular cell proliferation, not only in normal kidneys but also in cyst formation and enlargement. Activation of the intrinsic kinase domain results in phosphorylation of specific tyrosine residues within the cytoplasmic tail [105] that serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signaling pathways [105,106]. The duration and strength of intracellular signaling of ErbB receptors depend on speed of down-regulation by internalization, as well as the ratio of receptors that are targeted for recycling or degradation. Homodimers of EGFR have a signal which does not last long, in part due to receptor downregulation and degradation after ligand-dependent activation [107]. EGF is a well-known mitogen for normal renal epithelia, and has been shown to hyper-stimulate proliferation in ADPKD and ARPKD cystic epithelia [13,108]. As mentioned above, EGF and EGF-reactive peptide species are secreted into the apical medium of cultured ADPKD epithelia, and high, potentially mitogenic concentrations have been measured in cyst fluids collected from ADPKD patients [6,13]. Increased expression of TGFα has been observed in PKD cells compared with normal kidney cells [109] and transgenic mice overexpressing TGFα develop cystic kidneys [110]. Abnormal expression of amphiregulin and heparin-binding EGF (HB-EGF) has also been reported in both autosomal dominant and autosomal recessive PKD [13,108–110]. In non-orthologous murine models of ARPKD HB-EGF showed the most marked activation of EGFR and was seen on apical membranes of cystic epithelia, while amphiregulin was ineffective and not on apical membranes. These studies suggested that the relative potencies of growth factors in the context of cyst expansion are a function of membrane ligand bound/receptor trafficking, as well as ligand availability [111].

Ligand-induced activation of ErbB receptors can stimulate a varety of intracellular pathways including PKC/AKT, PLCγ, MEK/Erk or c-Src. Phosphorylation of EGFR at Y845 in the kinase domain is implicated in stabilizing the activation loop, maintaining the active state of the enzyme and providing a binding surface for substrate proteins. c-Src binds to and

phosphorylates EGFR at Y845 and is required for ErbB family-mediated signaling and proliferation in normal cells as well as in cystic renal epithelia [104,112,113]. It was also shown that increased pY418-Src activity correlated with cystic expansion in BPK mice and PCK rats and that inhibition of Src activity could attenuate of renal cystic disease in these respective phenotypic and genotypic rodent models of ARPKD [104].

Over-expression, constitutive activation and abnormal location of EGFR and ErbB2 receptors on the apical (luminal) surface of cyst-lining epithelia, together with secretion of soluble ligands by PKD cyst lining epithelia, creates a sustained cycle of autocrine–paracrine stimulation of proliferation in cysts [13,100,102,114]. A precisely controlled balance between cellular proliferation and apoptosis is essential for normal growth and differentiation of the kidney and maintenance of normal renal structure. These fundamental processes are disturbed in cystic kidneys [6,115]. Moreover, it was shown recently that EGFR membrane polarity is regulated by multiple hierarchical sorting pathways in renal epithelial cells and some of these pathways are perturbed in the BPK ARPKD mouse model [116].

Another common feature in PKD kidneys is a failure to switch off fetal genes and proteins, such as ErbB2, after completion of the developmental period [187] ErbB2 receptor which is highly expressed in several human cancers, including renal carcinomas, as well as in PKD, can potentiate EGFR signaling by slowing down the internalization and degradation of EGFR [117]. Although ErbB2 requires heterodimerization with another ligand-bound ErbB family member, such as EGFR, to be activated by autophosphorylation at Y1248 and Y1221/1222 [118], heterodimers are not only the favored complexes. As noted above, activation of ErbB2 is known to produce the strongest ErbB-related intracellular signaling responses. The importance of the ErbB2-receptor variant is further demonstrated both in cultured cells, where overexpression and activation of ErbB2 enhances proliferation and migration [119–122] and in transgenic mice where overexpression of ErbB2 induces renal cyst formation [108,123].

7. EGF-mediated regulation of sodium transport in normal and PKD cells

The epithelial Na^+ channel (ENaC) is a member of the ENaC/Deg channel superfamily [124–128]. ENaC activity is the rate limiting step for Na^+ (re)absorption across many epithelia, including those in the distal nephron, lungs and colon. Dysfunction and aberrant regulation of this channel leads to a spectrum of diseases ranging from hyper- and hypotension associated with improper renal Na^+ conservation and wasting, respectively, to respiratory syndromes linked to cystic fibrosis [129,130]. Moreover, several forms of congenital hypo- and hypertension, such as pseudohypoaldosteronism and Liddle's syndrome, respectively, result from ENaC dysfunction with the prior being caused by loss of function mutations in the channel and the latter by gain of function mutations [131].

EGF-family ligands regulate various epithelial channels, including ENaC, TRPC5, TRPM6 and PC-2. As we discussed above, EGF and it related growth factors have direct actions on the primary route of Na^+ reabsorption in the distal nephron. This site of the nephron gives rise to many of the cystic epithelial cells in PKD particularly in ARPKD which appears to be limited to distal tubular cystic development [6,10,132–134]. Activity of ENaC is the rate limiting step in Na^+ reabsorption at this site [125,126,128]. Thus, ENaC dysfunction has been linked to a broad spectrum of human diseases including PKD where it may have an important pathophysiological role at the very earliest stages of the disease. Here we will summarize recent advances in the understanding of regulation of epithelial ion channels by EGF-family ligands in the normal and cystic kidney. This review will certainly not be complete, in part due to the considerable bulk of data and to the recent burst of new findings

on this topic, and we apologize in advance to the authors of any relevant studies that we fail to mention

7.1. Role of EGF in regulation of ion channels in the kidney

EGF and its related growth factors are involved in regulation of various epithelial ion channels in the kidney. For instance, EGF stimulates store-operated Ca^{2+} channels in human mesangial cells through an intracellular signaling mechanism involving tyrosine kinase and PKC [135]. Later it was shown that EGF activates store-operated Ca^{2+} channels by a PLC-dependent but IP_3 receptor independent pathway [136].

The TRP superfamily of signal-transduction-gated ion channels is widely expressed in the kidney [79,137–139]. Bezzerides et al. have shown that EGF stimulation rapidly induces TRPC5 channel translocation to the plasma membrane. The small G protein Rac1 mediated the rapid insertion of TRPC5 into the plasma membrane through stimulation of PI(4)P 5-kinase, and increased channel availability. The authors proposed that rapid insertion of TRP channels may be a general process of cells in response to stimuli that affect cell morphology and perhaps, direction of movement [140]. We have also shown that RhoA and Rac1, small G proteins in the Rho family affect ENaC trafficking [25,141–144]. However, a role of growth factors in Rho-mediated increase of ENaC activity is not clear yet.

A role for ErbB receptor activation in the regulation of Mg^{2+} channels in the kidney has also recently been proposed. The regulation of magnesium in the body principally occurs within the kidney. Genetic analysis has revealed that the TRPM6 channel is mutated in patients with primary hypomagnesemia and secondary hypocalcemia [145,146]. Moreover, Groenestege et al., described a mutation in the EGF gene, encoding pro-EGF, which is responsible for a rare form of renal Mg^{2+} wasting, isolated recessive hypomagnesemia [147]. TRPM6 is a magnesium channel that is expressed in the intestine and renal distal tubules. Two different laboratories studied the effects of EGF on TRPM6 channels. Miwa and colleagues have shown that EGF increased the phosphorylation of ERK1/2 and TRPM6 expression in renal epithelial NRK-52E cells. Furthermore, EGF enhanced the influx of magnesium. Thus it was proposed that the phosphorylation of ERK1/2 may up-regulate TRPM6 expression and magnesium influx, resulting in an increase in cell proliferation with a shift from G1 to S phase [148]. Recently they have shown that the ERK/AP-1-dependent pathway is involved in the transcriptional regulation of TRPM6 expression [149]. Bindels and colleagues reported that EGF-mediated stimulation of TRPM6 occurs *via* signaling through Src kinases and the small GTPase Rac1, thereby redistributing endomembrane TRPM6 to the plasma membrane [150]. It is interesting that this effect was specific since stimulation of the EGFR increased current through TRPM6 but not TRPM7 [150]. Recent study showed that EGFR inhibitor erlotinib is capable of affecting TRPM6 regulation and thereby altering Mg^{2+} handling *in vivo*. Erlotinib can influence Mg^{2+} handling but its effect on the systemic Mg^{2+} concentration seems less potent than that observed with antibody-based EGFR inhibitors. Thus these data suggest that typical human dosages of erlotinib are unlikely to severely affect serum Mg^{2+} concentrations [151].

7.2. EGF-mediated regulation of polycystins

PC-2 can be activated in response to EGF in the kidney epithelial cell line LLC-PK$_1$ [152]. PC-2 overexpression increases EGF-induced inward currents in LLC-PK$_1$ cells, while the knockdown of endogenous PC-2 by sRNAi or the expression of a pathogenic missense variant, PKD2-D511V, blunts the EGF-induced response [152]. The physiological relevance of EGF-induced activation of TRPP2 in LLC-PK$_1$ cells was supported by whole animal studies in which homozygous deletion of the *EGFR* gene resulted in cystic dilatation of collecting ducts [70,153]. EGF-induced activation of PC-2 occurred independently of store

depletion but required the activity of phospholipase C (PLC) and phosphatidylinositol 3-kinase (PI3-kinase). Interestingly, PC-2 interacted with PLC-γ2 and colocalized in the primary cilium with EGFR and PIP$_2$ [152]. The authors proposed that PC-2 is under negative regulation by PIP$_2$, which can be reversed by EGFR activation.

In addition, yeast two-hybrid screen analysis identified mDia1 (mammalian Diaphanous-related formin 1 protein) as a PC-2-interacting protein [154]. mDia1 is a member of the small G protein RhoA-binding-formin protein family that participates in cytoskeletal organization, cytokinesis, and signal transduction. mDia1 functioned as an intracellular, voltage-dependent regulator of PC-2. At resting potentials, autoinhibited mDia1 bound to and blocked PC-2, whereas at positive potentials activated mDia1 released the block on PC-2 leading to channel activation. EGF or membrane depolarization activated PC-2 through the sequential activation of RhoA and mDia1. The voltage-dependent block of PC-2 by mDia1 served two physiologically relevant roles, to underlie the activation mechanism of PC-2 by EGF and to set the resting membrane potential of kidney epithelial cells to its resting value by preventing constitutive activation of PC-2 [155].

7.3. EGF and its related growth factor-dependent regulation of ENaC-mediated sodium transport in principal cells

EGF has been known for almost two decades to regulate sodium transport. However, several investigations have reported contradictory results indicating opposite effects of EGF on ENaC activity and sodium transport. EGF appears to affect sodium transport and ENaC in a context-dependent manner: it increases sodium absorption in the airways [156] and intestine [157], but decreases sodium transport in the isolated and perfused rabbit CD [158–161], immortalized murine CD mCT1 cells [162,163], in cystic fibrosis JME/CF15 cells [164], in Chinese hamster ovary (CHO) cells overexpressing ENaC subunits and EGFR [165] and in Madin-Darby canine kidney cells [166,167]. Markadieu et al. demonstrated that EGF transiently increases sodium transport in Xenopus laevis kidney A6 cells in a phosphatidylinositol 3-kinase (PI 3-kinase)-dependent manner, and that this stimulation is increased by the inhibition of the MAPK pathway [168].

Recently Liu et al. have proposed that ENaC shows a biphasic response to EGF and TGF-α in A6 cells [169]. These authors also showed that acute treatment with EGF and TGF-α enhanced ENaC activity by increasing P_o and that PI 3-kinase was involved in the activation of sodium transport [169]. Similarly, EGF via the small G protein Rac1, acutely activates ENaC in alveolar epithelial cells [170]. However, chronic treatment with EGF and TGF-α inhibited ENaC activity by decreasing the number of channels (*N*) in the plasma membrane through MAPK1/2 pathways [169]. Short-term blockade of the MAPK1/2 has also been shown to down regulate both the basal Na$^+$ current and the aldosterone and vasopressin stimulated Na$^+$ current in mpkCCD$_{c14}$ principal cells. The authors proposed that the activity of Na, K-ATPase but not that of ENaC was inhibited by MEK1/2 inhibitors in both unstimulated and aldosterone- or vasopressin-stimulated CCDs [171]. Similarly, inhibitors of the MAPK1/2 cascade, protected Na$^+$ reabsorption from PKC, demonstrating that the MAPK1/2 cascade, in some instances, plays a central role in down-regulation of ENaC activity [172]. Furthermore, we have shown that the small G protein K-Ras activates ENaC activity via PI 3-kinase in both heterologous and native principal cells [173–175]. K-Ras is an aldosterone-induced protein and most likely its effect on ENaC is mediated by this hormone [176–179]. However, K-Ras expression and activity could also be regulated by growth factors such as EGF [180,181]. Furthermore, it was proposed that EGFR is transactivated by aldosterone [182]. For example, it was shown recently that aldosterone-induced mesangial cell proliferation is mediated by EGFR transactivation which was dependent on the K-Ras/c-Raf/MEK/ERK and PI 3-kinase/Akt/mTOR/p70S6K1 signaling

pathways [183]. However it is still remains to be shown whether K-Ras or other small G proteins are involved in EGF-mediated regulation of ENaC activity.

Our recent data are consistent with the idea that EGF and its related growth factors (TGF-α, HB-EGF and amphiregulin) have a biphasic effect on sodium absorption in the mammalian kidney as represented by mpkCCD$_{c14}$ cells and that inhibition of MEK1/2 stimulates acute increase and abolishes chronic decrease of sodium transport in response to EGF action [23]. As shown in Fig. 3, addition of all four studied EGF family growth factors to polarized mpkCCD$_{c14}$ principal cells with steady-state basal transport rates significantly increased Na$^+$ reabsorption above basal levels in a time-dependent manner. Significant increases were detectable after 30 minutes, the earliest time point measured, with a maximum reached by 2 hours. After 4 hours of stimulation, a slow and continuous decrease of I$_{sc}$ (short circuit current) was observed. Chronic treatment of monolayers with EGF and its related growth factors (added basolaterally) leads to significant inhibition of I$_{sc}$. As illustrated in Fig. 3, addition of the Na$^+$ channel inhibitor amiloride (10 μM) at the end of experiments to the apical bathing solution caused a rapid decrease in I$_{sc}$. Nearly all of the basal I$_{sc}$ was inhibited by amiloride. Thus, the addition of EGF caused a transient increase in I$_{sc}$ followed by a chronic decline in I$_{sc}$ and these effects were mediated by receptors located at the basolateral membrane. The acute effect of EGF was mediated via PI3-kinase and both phases were mediated by MEK1/2. Acute effect is most likely mediated by changes in the channel's open probability and long term effect occurred due to the decrease of the number of active channels at the plasma membrane [23,169]. Furthermore, our study underlined the critical role of ErbB2 in the regulation of sodium transport in these collecting duct cells and suggested that EGF influences ENaC and sodium absorption via the ErbB2 receptor, most probably by forming ErbB2/EGFR heterodimers [23]. Fig. 4 shows our favored possible model for regulation of ENaC in normal CD monolayers by EGF-family ligands. As demonstrated in this figure, there are at least two independent pathways involved in the regulation of ENaC. However, it is very likely that other proteins are involved in regulation of ENaC. As we discussed previously, Bezzerides et al. demonstrated that EGF stimulation rapidly induces TRPC5 channel translocation to the plasma membrane and Rac1 mediates this insertion[140]. Bindels and colleagues also reported that EGF-mediated stimulation of TRPM6 occurs via signaling through Src kinases and Rac1 [150]. Importantly, Fujita and colleagues have established that signaling cross-talk between Rac1 and the mineralocorticoid receptor modulates receptor activity and identified Rac1 as a therapeutic target for chronic kidney disease[184,185]. Garvin's and Helms' laboratories also recently identified an important role for Rac1 in NaCl-induced superoxide generation in the medullary thick ascending limb[186] and in the alveolar epithelium[170]. Thus, small GTPase Rac1 is a probable effector of EGF and its related growth factors with respect to ENaC activity. However this hypothesis requires further studies.

7.4. Regulation of sodium transport in PKD cells

Since EGF and its related growth factors regulate ENaC activity, it is possible that ENaC inhibition facilitates cyst formation in PKD [187]. Considerable experimental data support the idea that there is coordinated interaction between ENaC and the cystic fibrosis transmembrane conductance regulator Cl$^-$ channel (CFTR) suggesting the possibility that ENaC may play some role in cystic fibrosis and other disease processes associated with dysfunctional CFTR [188–192]. Increased airway Na$^+$ absorption *in vivo* caused airway surface liquid volume depletion, increased mucus concentration, delayed mucus transport and mucus adhesion to airway surfaces. Defective mucus transport caused a severe spontaneous lung disease sharing features with cystic fibrosis [193]. The airway surface liquid volume modulates the activity of ENaC by modification of the serine protease-protease inhibitor balance and alterations in this balance contribute to excessive Na$^+$

absorption in cystic fibrosis [194]. It was shown that the CFTR Cl^- channel exists in apical membranes of human ADPKD cells and may play an important role in cyst formation or enlargement [195]. Cl^- currents in cultured ADPKD cells were activated by forskolin and cAMP and had properties identical to those that characterized CFTR [195]. Later Magenheimer et al. have shown that cAMP-stimulated fluid secretion occurs early in embryonic renal tubule development in wild-type and PKD kidneys at the time when renal cysts first appear in ADPKD, suggesting that a cAMP-driven mechanism may be involved in the initial stages of cyst formation in ADPKD [196]. Moreover, CFTR inhibitors partially inhibited cyst growth and preserved renal function in a mouse model of ADPKD [197]. However, renal cyst formation in a murine model of ARPKD did not require functional activity of CFTR [198].

In ADPKD, all the segments of the nephron are involved in forming the fluid-filled cysts. In ARPKD, dilated tubules rather than isolated cysts are predominantly formed from the CD, which is the nephron segment where ENaC plays a major role in the regulation of sodium transport [199,200]. ENaC dysfunction associated with improper handling of NaCl has been demonstrated in renal epithelial cells from animal models and human ARPKD cell lines [201–204].

Measurements of transepithelial ^{22}Na transport performed on monolayers of ARPKD and age-matched human fetal collecting tubule (HFCT) cells revealed net Na^+ absorption in both models. However, ARPKD cells absorbed Na^+ at a rate approximately 50% greater than that of HFCT. Furthermore, northern blot analyses of ARPKD whole kidney and western immunoblot of ARPKD cells showed approximately two fold greater expression of the α-ENaC. These results suggest that ARPKD cyst-lining cells absorb Na^+ by a pathway that involved ENaC activity [205]. Later it was shown that ENaC-mediated Na^+ absorption is upregulated in a murine ARPKD model epithelium lacking apical monocilia [202]. Three- to fourfold elevated Isc was measured in mutant *orpk* (Oak Ridge PKD) monolayers versus rescued controls. Examination of this enhanced end point in mutant monolayers revealed that that this effect was mediated by ENaC. These data suggested that ENaC expression and/or function are upregulated in the apical membrane of mutant *orpk* CD principal cell monolayers [202]. However, Veizis et al. have shown that amiloride sensitive Na^+ absorption is decreased in CD cells from the non-orthologous BPK mouse model of ARPKD [203]. Moreover, they present evidence that addition of EGF to the apical bathing solution (24 hrs) led to inhibition of Na^+ transport and decreased ENaC expression in cystic cells and that this effect was mediated through ERK kinase [204]. Thus the authors suggest that the mislocalized apical ErbB receptors are functionally coupled to the ERK pathway and that abnormal EGF-dependent regulation of ENaC function and expression may contribute to PKD pathophysiology [204].

Thus, role of ENaC-mediated sodium absorption and its regulation by EGF in PKD is obvious. However, several investigations have reported contradictory results. Thus, the exact mechanism by which ENaC-mediated sodium transport involved in development and progression of PKD, and especially ARPKD, is not completely clear. As indicated by Guay-Woodford [134], although identified abnormalities in the tubular epithelia diverse and not necessarily reproducible from one model to another, these abnormalities provide the framework for evaluating interventions that target specific processes and pathways involved in PKD pathogenesis. Hence investigation of the modulation of sodium transport by growth factors in PKD models would facilitate a more complete understanding of the pathologic development of PKD. Additional studies using cells with endogenous genotypic and phenotypic effects of PC-1, PC-2 and Fibrocystin-1 mutations will be required to understand the role of ENaC in PKD and EGF-mediated regulation of sodium transport in PKD. Our preliminary immunohistochemistry analysis revealed that ENaC subunits are expressed on

the apical surface of CDs in PCK rats, the genotypic model of ARPKD. Moreover, we were able to record ENaC activity with patch clamp electrophysiology (unpublished observations) in freshly isolated opened cysts from the PCK rats. However, these observations require further investigations.

8. Clinical Relevance

ErbB family members are implicated in the development of cardiac and renal diseases such as cancer, hypertension and ESRD. Therefore, the therapeutic potential of targeting of ErbB receptors and EGF-family signaling pathways may play key roles in the kidney. A number of studies have investigated the potential role of EGF and its related growth factors in acute renal injury and both ADPKD and ARPKD [4,6,21,58]. Addition of ErbB receptor ligands to epithelial cells promotes cell proliferation, migration, and epithelial-mesenchymal cell transdifferentiation. Chronic ErbB activation and mislocalization are thought to be important in the evolution of PKD. The cyst fluid of PKD patients contains mitogenic concentrations of ErbB receptor ligands [109,206–208]. Thus this might represent a protective mechanism of diseased kidney in PKD.

ADPKD and ARPKD have overlapping but distinct pathogeneses. Identification of the causative mutated genes and elucidation of the function of their encoded proteins and signaling mechanisms mediating their regulation will shed new light on these diseases. Improved understanding of pathogenesis and mechanisms involved in both ADPKD and ARPKD provide an excellent opportunity for the development of pathophysiology-based therapies. Some of these have proven effective in preclinical studies, and clinical trials have begun [59,209]. Our enhanced understanding of the PKD phenotype at a cellular level will facilitate identification of other interventional targets.

9. Conclusion

The study of regulation of ion channels and proliferation by ErbB receptor ligands has expanded considerably in recent years. *In vitro* and *in vivo* studies have provided direct evidence of the role of ErbB signaling in the kidney. Collectively, these results suggest that ErbB receptors could be major determinants in the development of renal lesions, possibly through enhanced cell proliferation and regulation of sodium reabsorption. However it is not exactly clear yet how ErbB receptors ligands affect proliferation and sodium reabsorption in PKD.

Inappropriate expression, activation and downstream signaling of ErbB receptors, and particularly ErbB2, contributes to the pathological phenotype of PKD and suggest that inhibition of this pathway represents a promising therapeutic target in this disease. Undoubtedly, many questions remain regarding regulation of sodium reabsorption and proliferation in normal and PKD cells by ErbB receptors and their ligands. For example, our data demonstrate that the ErbB2 receptor is involved in ENaC-mediated sodium transport in mpkCCD$_{c14}$ principal cells. It is most likely that ErbB2 forms a heterodimer with EGFR, although a role for ErbB3 and ErbB4 cannot be excluded. Neuregulins-1 through -4, which can bind either both ErbB3 and ErbB4 or only ErbB4 could be utilized in future experiments to define any potential involvement of ErbB3 and ErbB4 in sodium transport. Furthermore, ErbB receptors after changing their polarity in PKD cells come into close proximity with ENaC and could thus potentially either form a complex with the channel or change its mechanisms of regulation. Future directions for study will require direct measurements of ENaC channel activity in response to ErbB-family ligands in genotypic ADPKD and ARPKD cell models.

Acknowledgments

Abbreviations

ADAM	a disintegrin and metalloprotease
Ang II	Angiotensin II
ADPKD	autosomal dominant polycystic kidney disease
ARPKD	autosomal recessive polycystic kidney disease
CCD	cortical collecting duct
CD	collecting duct
DCT	distal convoluted tubule
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENaC	epithelial Na^+ channel
ESRD	end-stage renal disease
GPCR	G-protein coupled receptor
HB-EGF	heparin-binding EGF
HFCT	human fetal collecting tubule
I_{sc}	short-circuit current
mDia1	mammalian Diaphanous-related formin 1 protein
MMPs	matrix metalloproteinases
PC-1	polycystin-1
PC-2	polycystin-2
PI 3-kinase	phosphatidylinositol 3-kinase
PKD	polycystic kidney disease
PLC	phospholipase C
PTB	phosphotyrosine binding domain
RTK	receptor tyrosine kinase
SD	Sprague-Dawley
SH2	Src homology domain 2
TAL	thick ascending limb
TGF-α	transforming growth factor alpha
TRP	transient receptor potential

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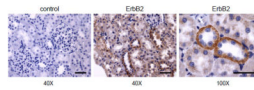
Fig. 1. ErbB2 expression in rat kidney tissue
Representative immunohistochemical staining for ErbB2 detection in kidney cortical sections of Sprague-Dawley rat. Original magnifications are 40× (b) and 100× (c). Scale bars are 50 µm. Negative control (a) (stained with secondary antibodies in the absence of primary antibodies) at 40× is also shown. The kidney was fixed for 24 hours in Zinc Formalin and processed for paraffin embedding as described previously [23,25]. The kidney sections were cut, dried and deparaffinized for subsequent labeled streptavidin-biotin immunohistochemistry. Tissue sections were incubated for 90 minutes in a 1:250 concentration of anti-ErbB2 (Cat. #ab2428; Abcam Inc., Cambridge, MA).
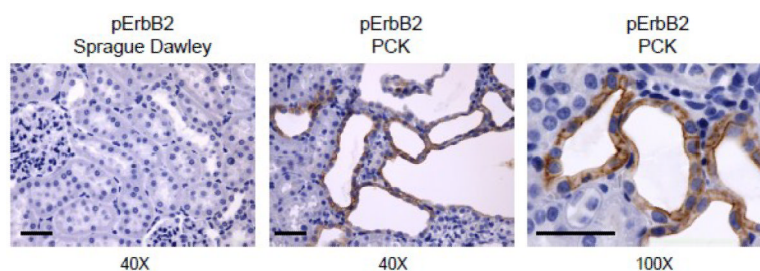
**Fig. 2. pErbB2 expression in normal and cystic rat kidney tissue**
Representative immunohistochemical staining for pErbB2 detection in kidney cortical sections of Sprague Dawley (a) and PCK (b, c) rats. Original magnifications are 40× and 100× (scale bars: 50 μm). Tissue sections were stained with anti-pErbB2 (Phospho-HER2/ErbB2 (Tyr1221/1222); 1:100; Cat. #2243; Cell Signaling Technology Inc., Boston, MA).
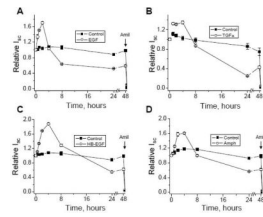
Fig. 3. Time course of biphasic effect of ErbB receptor ligands on ENaC-mediated sodium transport in mpkCCD$_{c14}$ principal cells

Summary graph of relative I$_{sc}$ in mpkCCD$_{c14}$ cells in response to EGF (10 ng/ml; a), TGF-α (10 ng/ml; b), HB-EGF (50 ng/ml; c) and amphiregulin (100 ng/ml; d). MpkCCD$_{c14}$ cells were serum-starved overnight. EGF-family ligands and vehicle (control) were added basolaterally at time 0 and current was normalized to the starting level. Amiloride (10 μM; arrows) was added to the apical membrane at the end of experiment. If error bars are not visible, they are smaller than the size of symbols. Values are means ± SE from at least 6 experiments. Some results represented here were previously published in a different format [23].
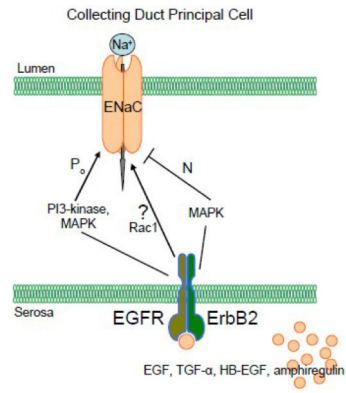
Fig. 4.
A schematic representation of EGF and its related growth factor-mediated regulation of ENaC activity in collecting duct principal cells.