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Anoctamin 1 induces calcium-activated chloride secretion and proliferation of renal cyst–forming epithelial cells

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Polycystic kidney diseases are characterized by multiple bilateral renal cysts that gradually enlarge and lead to a decline in renal function. Cyst enlargement is driven by transepithelial chloride secretion, stimulated by enhanced levels of cyclic adenosine monophosphate, which activates apical cystic fibrosis transmembrane conductance regulator chloride channels. However, chloride secretion by calcium-dependent chloride channels, activated through stimulation of purinergic receptors, also has a major impact. To identify the molecular basis of calcium-dependent chloride secretion in cyst expansion, we determined the role of anoctamin 1 and 6, two recently discovered calcium-activated chloride channels both of which are expressed in epithelial cells. We found that anoctamin 1, which plays a role in epithelial fluid secretion and proliferation, is strongly expressed in principal-like MDCK cells (PLCs) forming cysts within a collagen matrix, in an embryonic kidney cyst model, and in human autosomal dominant polycystic kidney disease tissue. Knockdown of anoctamin 1 but not anoctamin 6 strongly diminished the calcium-dependent chloride secretion of PLCs. Moreover, two inhibitors of anoctamin ion channels, tannic acid and a more selective inhibitor of anoctamin 1, significantly inhibited PLC cyst growth and cyst enlargement in an embryonic kidney cyst model. Knockdown of ANO1 by morpholino analogs also attenuated embryonic cyst growth. Thus, calcium-activated chloride secretion by anoctamin 1 appears to be a crucial component of renal cyst growth.

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Polycystic kidney diseases comprise a number of inherited disorders that lead to bilateral renal cyst development.[1] The most common form, autosomal dominant polycystic kidney disease (ADPKD), affects 1:400–1000.[1] ADPKD is characterized by continuous cyst enlargement over time, which leads to compression of adjacent normal parenchyma and often causes the need for renal replacement therapy.[1] Cyst growth is characterized by proliferation of the cyst-lining cells and fluid secretion across the epithelium into the cyst lumen.[2] Apical cyclic adenosine monophosphate (cAMP)-dependent chloride secretion is one of the major driving forces for cyst fluid secretion.[2] Data obtained with inhibitors of the cystic fibrosis transmembrane conductance regulator (CFTR) in various cyst models suggest that the CFTR chloride channel has an important role in this process.[3–5] However, there is significant heterogeneity in CFTR expression in cyst epithelia,[6,7] suggesting that other chloride channels may also be involved in cyst secretion. In line with this idea, activation of apical calcium-activated chloride channels following stimulation of Gq-coupled purinergic receptors (P2YR) has been described in the Madin-Darby canine kidney (MDCK) cyst model[8,9] and *ex vivo* in ADPKD cyst epithelia.[10]

The anoctamin ion channel family comprises 10 proteins (ANO1–10), which are probably all capable of mediating calcium-activated chloride currents.[11] Of all anoctamins, ANO6 is the most widely expressed paralog and has recently been demonstrated to produce chloride currents activated by high intracellular calcium, cell swelling, or apoptotic stimuli.[12,13] ANO1 has been identified as a calcium-activated chloride channel, which is expressed in a wide range of epithelial and nonepithelial cells.[14–16] It is highly expressed in kidneys, as well as in renal epithelial cell lines,[17,18] with a predominant function in epithelial cells.[18,19] In epithelial tissues from ANO1 knockout mice, calcium-dependent chloride secretion is significantly impaired.[12,19] Apart from prosecretory effects, upregulation of calcium-activated chloride channels has also been observed to have a role in proliferation.[20,21] Along these lines, ANO1, also known as DOG1, was found to be a reliable marker in gastrointestinal stromal tumors and head and neck squamous cell carcinoma, and its expression correlates with

poor outcome in esophageal squamous cell cancer.[22,23] Owing to its proproliferative role, targeting of ANO1 has been proposed for the treatment of malignant tumors.[24]

On the basis of these data, we hypothesized that anoctamins, in particular ANO1, may contribute to renal cyst formation in two ways: (i) by augmenting cell proliferation and (ii) by activating fluid secretion into the cyst lumen.

RESULTS

Stimulation of purinergic receptors in principal-like MDCK cells (PLCs) leads to calcium-activated chloride secretion

Recently, we have shown that only MDCK cells resembling principal cells from the collecting duct (PLCs) form cysts within a collagen 1 matrix and enlarge in the presence of forskolin, which leads to elevation of intracellular cAMP.[8] Furthermore, cAMP-dependent cyst growth was dependent on the presence of extracellular ATP, thus suggesting a synergy between cAMP- and calcium-dependent chloride secretion.[8] To better understand the molecular mediators of calcium-dependent chloride secretion in cyst-forming MDCK cells, we grew PLCs to high-resistance tight monolayers ($11{,}824 \pm 1397\,\Omega cm^2$; $n = 14$) and examined the transport activity of the epithelium in Ussing chamber experiments. The transport activity was low in nonstimulated resting monolayers, but it was strongly augmented by the activator of purinergic P2Y2 receptors, uridine triphosphate (UTP) (100 μmol/l). UTP induced negative voltage deflections when applied either to luminal or basolateral sides of the epithelium (Figure 1a). Calculation of the equivalent short-circuit current indicated a significant increase of chloride secretion by UTP (Figure 1b). A negative voltage deflection by increased chloride secretion was also induced by a direct increase in intracellular calcium concentration caused by the calcium ionophore ionomycin (1 μmol/l; Figure 1c and d). Chloride replacement by the use of gluconate almost completely abolished UTP-dependent currents, confirming that the currents were chloride dependent (Figure 1e and f). Significant voltage deflections and short-circuit currents were also obtained by the use of 10 μmol/l UTP or 10 μmol/l ATP, respectively (Supplementary Figure S1 online), representing concentrations that have been detected in the cyst fluid of ADPKD kidneys.[25] UTP-induced chloride secretion was inhibited by 10 μmol/l clotrimazole, an inhibitor of calcium-dependent SK4 K^+ channels, which provides the driving force for chloride secretion by hyperpolarizing the membrane voltage. Moreover, common inhibitors of calcium-activated chloride channels, such as niflumic acid (10 μmol/l), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (100 μmol/l), or tannic acid (TA; 10 μmol/l),[26] inhibited UTP-induced chloride secretion (Figure 1g).

Knockdown of ANO1 abolishes calcium-dependent chloride secretion in PLCs

Next, we investigated the expression of ANO1 to ANO10 mRNA in PLCs by reverse transcriptase–PCR (RT–PCR). Besides ANO1 and ANO6, ANO 3, 4, 8, and 9 were expressed in PLCs (Supplementary Figure S2A online). As ANO1 is expressed in a wide range of epithelial cells[14–16] and ANO6 is the most widely expressed paralog,[11] we focused on the role of ANO1 and ANO6 for calcium-dependent chloride secretion in cyst-forming PLCs. We transiently transfected PLCs with small interfering RNA (siRNA) directed against ANO1 or ANO6. Successful downregulation of ANO1 or ANO6 mRNA by siRNA treatment was demonstrated by real-time RT–PCR (Supplementary Figure S2B and C). As demonstrated in Figure 2, knockdown of ANO1 but not ANO6 almost completely abolished calcium-activated chloride secretion in these cells (Figure 2a). Moreover, knockdown of ANO1 largely attenuated activation by either luminal or basolateral application of UTP (Figures 2b and c). Ionomycin-activated chloride secretion was also inhibited by siRNA-ANO1 but not siRNA-ANO6 (Figure 2d). As expected, the inhibitory effects of clotrimazole and various blockers of calcium-activated chloride channels were largely reduced after siRNA knockdown of ANO1 but not of ANO6 (Figure 2e). On the basis of these data indicating a functional role of ANO1 for calcium-activated chloride secretion, we then investigated the expression of ANO1 in the cyst-lining epithelium.

ANO1 is expressed in the cyst-lining epithelium of two different cyst models and human ADPKD tissue

We stained cysts of two different *in vitro* cyst models and human ADPKD tissue for ANO1 using two affinity-purified antibodies recognizing two different epitopes of ANO1, one of which binds to a highly homologous sequence in mice and dogs.[18,27] ANO1 knockout tissue served as the control (Supplementary Figure S3B online).

Nonstimulated cysts derived from PLCs grown within a collagen 1 matrix showed a diffuse staining for ANO1, including subtle signals in the apical membrane and robust signals in the cytoplasm (Figure 3a and b). Stimulation of PLC cysts with forskolin led to a significant increase of ANO1 staining in the luminal membrane, accompanied by a decrease of the cytosolic signal (Figure 3a and c). This may point toward a cAMP-dependent trafficking of ANO1 toward the apical membrane and is in line with previous data showing synergistic effects of cAMP- and calcium-dependent chloride secretion.[8]

Metanephric kidneys from embryonic days E12.5–E13.5 were cultured for 5 days, and forskolin stimulation was started from day 2 *ex vivo*. As demonstrated earlier, stimulation with forskolin induces tubular dilation, followed by cyst formation, and cyst growth with secretion mechanisms resembling those *in vivo*.[4,5] In line with the data from the PLC cyst model, cyst-lining cells of many, albeit not all cysts, showed apical staining for ANO1 (Figure 3d and e). Notably, there is a change of ANO1 expression during kidney development. Nonstimulated kidneys at E12.5 showed ubiquitous staining for ANO1 comprising the apical and basolateral membrane of tubular cells and a diffuse staining of interstitial cells (Supplementary Figure S4 online). In contrast, in kidneys dissected at E12.5 and grown *ex vivo* for 5 days, ANO1 was found predominantly in tubular cells

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