Biol. Chem., Vol. 392, pp. 125–134, January/February 2011 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2011.010

Review

Role of the Ca^{2+}-activated Cl^- channels bestrophin and anoctamin in epithelial cells

Karl Kunzelmann[1,*], Patthara Kongsuphol[1], Krongkarn Chootip[2], Caio Toledo[1], Joana R. Martins[1], Joana Almaca[1], Yuemin Tian[1], Ralph Witzgall[3], Jiraporn Ousingsawat[1] and Rainer Schreiber[1]

[1] Institut für Physiologie, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany
[2] Naresuan University, Phitsanulok 65000, Thailand
[3] Institut für Anatomy, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany

*Corresponding author
e-mail: karl.kunzelmann@vkl.uni-regensburg.de

Abstract

Two families of proteins, the bestrophins (Best) and the recently cloned TMEM16 proteins (anoctamin, Ano), recapitulate properties of Ca^{2+}-activated Cl^- currents. Best1 is strongly expressed in the retinal pigment epithelium and could have a function as a Ca^{2+}-activated Cl^- channel as well as a regulator of Ca^{2+} signaling. It is also present at much lower levels in other cell types including epithelial cells, where it regulates plasma membrane localized Cl^- channels by controlling intracellular Ca^{2+} levels. Best1 interacts with important Ca^{2+}-signaling proteins such as STIM1 and can interact directly with other Ca^{2+}-activated Cl^- channels such as TMEM16A. Best1 is detected in the endoplasmic reticulum (ER) where it shapes the dynamic ER structure and regulates cell proliferation, which could be important for renal cystogenesis. Ca^{2+}-activated Cl^- channels of the anoctamin family (TMEM16A) show biophysical and pharmacological properties that are typical for endogenous Ca^{2+}-dependent Cl^- channels. TMEM16 proteins are abundantly expressed and many reports demonstrate their physiological importance in epithelial as well as non-epithelial cells. These channels are also activated by cell swelling and can therefore control cell volume, proliferation and apoptosis. To fully understand the function and regulation of Ca^{2+}-activated Cl^- currents, it is necessary to appreciate that Best1 and TMEM16A are embedded in a protein network and that they probably operate in functional microdomains.

Keywords: anoctamin 1; bestrophin; Ca^{2+}-activated Cl^- currents (CaCCs); cancer; cystic fibrosis; purinergic receptors; TMEM16A; TMEM16B.

Introduction

Ca^{2+}-activated Cl^- currents (CaCCs) are abundant and present in almost any cell types, although with slight differences regarding their biophysical properties and pharmacology (Hartzell et al., 2005a). In many cases these currents modify the cellular response to adequate stimuli and therefore the amenable channels could be ideal pharmacological targets. Diverse functions such as olfactory-, taste- and phototransduction; neuronal and cardiac excitability; smooth muscle contraction and endothelial function, as well as fertilization and epithelial Cl^- secretion are controlled by CaCCs (reviewed in Frings et al., 2000; Kidd and Thorn, 2000; Eggermont, 2004; Leblanc et al., 2005; Melvin et al., 2005; Hartzell et al., 2005a) (Figure 1). Two families of proteins were shown to function as Ca^{2+}-activated Cl^- channels or to be major components of it: bestrophins and TMEM16 (anoctamin). The properties of both types of ion channels have been reviewed in several recent papers (Hartzell, 2008; Hartzell et al., 2008; Kunzelmann et al., 2009; Duran et al., 2010). For Best1 it has been an issue whether they are actually Cl^- channels or whether they control intracellular Ca^{2+} levels (Marmorstein et al., 2006; Hartzell, 2008; Barro Soria et al., 2009a). As outlined below, a possible answer to this question has meanwhile been provided (Barro Soria et al., 2009a; Neussert et al., 2010). In contrast, few doubts exist that TMEM16A is a major component of the plasma membrane localized Ca^{2+}-activated Cl^- channel (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008).

Best1 is a Cl^- channel and a regulator of channel activity

Numerous studies demonstrate the Cl^- channel properties of Best1, which is expressed at high levels in retinal pigment epithelial (RPE) cells (Marmorstein et al., 2000; Sun et al., 2001; Qu et al., 2003; Tsunenari et al., 2003; Qu and Hartzell, 2004; Yu et al., 2006). The so-called light peak in the electrooculogram is presumably generated by Ca^{2+}-dependent activation of Cl^- channels in the basolateral membrane of the retinal pigment epithelium and is reduced in patients with autosomal dominant vitelliform macular dystrophy caused by mutations in Best1 (Best disease; OMIM 607854) (Petrukhin et al., 1998; Hartzell, 2008). It was proposed that Best1 is the Ca^{2+}-activated Cl^- channel in the basolateral membrane of RPE cells, although several reasons argue against such a role (Hartzell et al., 2005b). Most important,

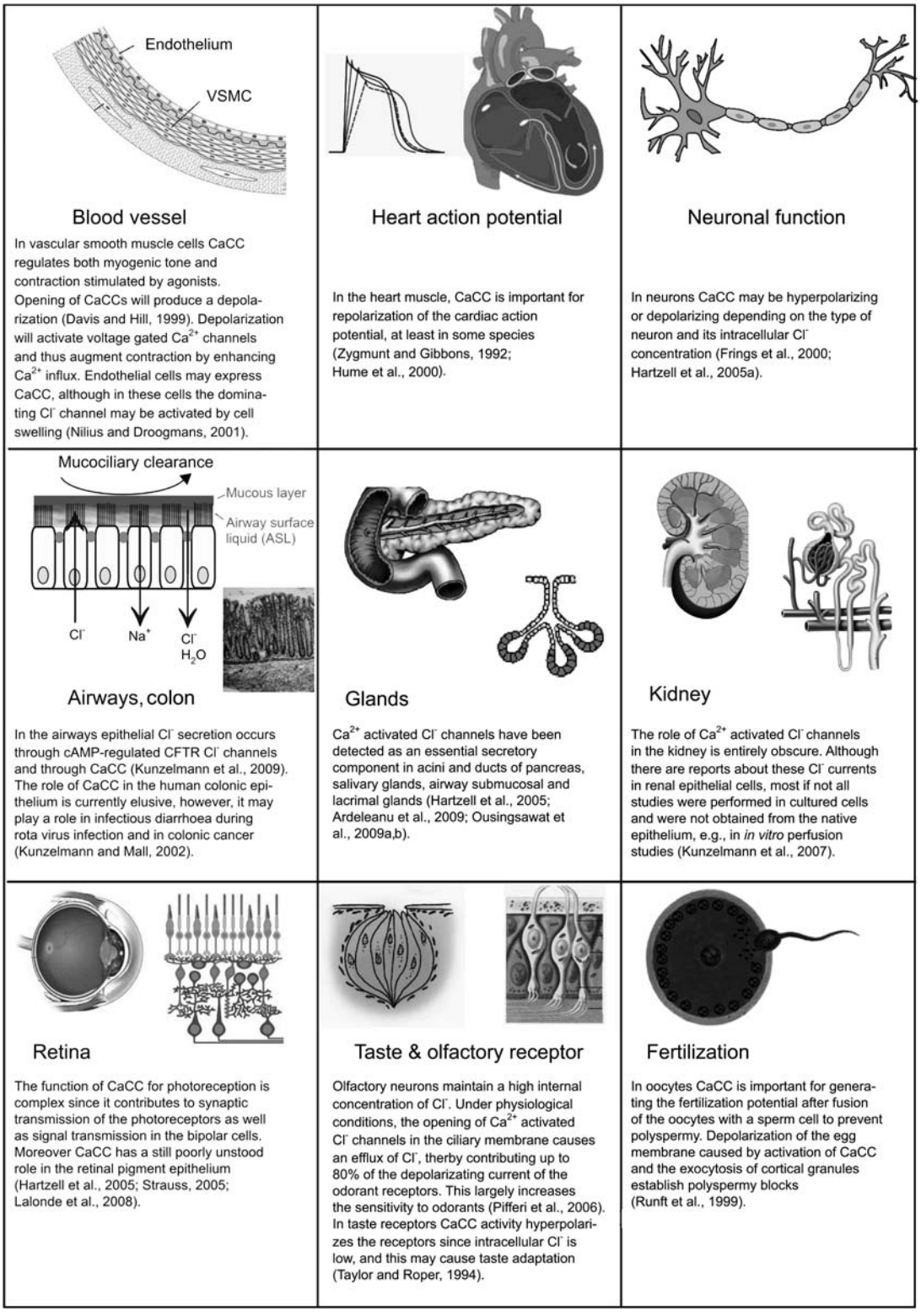

Figure 1 Tissue functions of CaCCs.

CaCCs are not absent in tissues of Best1 knockout mice (Marmorstein et al., 2006; Marmorstein and Kinnick, 2007; Barro Soria et al., 2008). However, human Best1 has been shown to change properties of voltage-gated Ca^{2+} channels in a RPE cell line (Rosenthal et al., 2006) and in HEK293 cells (Yu et al., 2008). Thus, the cellular role of Best1 in RPE cells, its role for the light peak and for macular dystrophy remains obscure. Several but not all recent studies con-

firm the role of Best1 for CaCCs, and also an association with the endoplasmic reticulum (ER) has been found (Matchkov et al., 2008; O'Driscoll et al., 2008, 2009; Neussert et al., 2010). Thus, Best1 seems to be both a Cl^- channel and a regulator of other ion channels (Rosenthal et al., 2006).

An intracellular function of Best1

How could Best1 operate as both a Cl^- channel and a regulator of Ca^{2+} signaling? We recently demonstrated that Best1 is localized in the ER in heterologous cells as well native epithelial cells (Barro Soria et al., 2009a). Best1 interacts with stromal interacting molecule 1 (Stim1), the ER-Ca^{2+} sensor. We found that intracellular Ca^{2+} transients elicited by stimulation of purinergic $P2Y_2$ receptors in HEK293 cells were augmented by expression of hBest1. Notably, the p21-activated protein kinase Pak2, which is known to play a role in both proliferation and apoptosis, was found to phosphorylate hBest1. Moreover, injection of active Pak2 (but not Pak1) activated a Cl^- conductance (Barro Soria et al., 2009a). This enhances intracellular Ca^{2+} signals and supports activation of truly membrane localized Ca^{2+}-dependent Cl^- (TMEM16A) and K^+ (SK4) channels. Furthermore, we found that lack of Best1 expression in respiratory epithelial cells of mBest1 knockout mice caused expansion of ER cisterns and induced Ca^{2+} deposits. As similar sites are present in both mBest1 and hBest1, bestrophins can share the same functions in mouse and human cells, despite differences reported earlier (Xiao et al., 2010). We therefore propose that Best1 is important for Ca^{2+} handling of ER stores and could resemble the long suspected counter ion channel to balance transient membrane potentials occurring through inositol trisphosphate (IP_3)-induced Ca^{2+} release and store refill. As Best1 can regulate compartmentalized Ca^{2+}-signaling, this could be important in Best macular dystrophy, inflammatory diseases such as cystic fibrosis or proliferation (Al Dehni et al., 2009; Barro Soria et al., 2009a).

Expression of the Best1 mutant R218C did not produce a substantial Cl^- current but caused an increased Ca^{2+} plateau when expressed in HEK293 cells, suggesting a role of Best1 for Ca^{2+} uptake into the ER (Barro Soria et al., 2009a). Notably, RPE cells of Best1-deficient mice showed higher levels of resting $[Ca^{2+}]_i$ than cells from wild-type mice and recovery from ATP-induced increase in $[Ca^{2+}]_i$ was delayed (Neussert et al., 2010). Zhang and coworkers detected a change in Ca^{2+}-signaling in RPE cells and also in knock-in models such as Best1(+/W93C) and Best1(W93C/W93C) (Zhang et al., 2010). Although, at present, results obtained in different cell types from the various mouse models are not entirely consistent, it is, however, obvious that Best1 changes intracellular Ca^{2+}-signaling. A proportion of endogenously expressed Best1 was localized to the ER where it influenced uptake of Ca^{2+} into Ca^{2+} stores. The authors suggested a role of Best1 as a Cl^- counter ion channel for Ca^{2+} uptake into cytosolic Ca^{2+} stores (Neussert et al., 2010). We recently expressed R218C-Best1 in Cos-7 cells, which show a high level of endogenous Best1 expression (data not shown). Expression of R218C-Best1 tagged with a tetracysteine and GFP was visualized in electron microscopy images by using the chromophore ReAsH which causes precipitation of the electron dense diaminobenzidine (DAB) after photoactivation (Griffin et al., 1998). With this method, R218C-Best1 was shown to be localized in the ER membrane (dark membrane staining, Figure 2A–C). Additionally expressing R218C-Best1 in Cos-7 cells demonstrates a remarkable change in ER appearance, from the typical tubular ER towards a vesicular structure (Figure 2A–C). As the ER is a highly dynamic structure, it can appear as organized smooth ER (OSER) in different shapes (Borgese et al., 2006).

These changes observed in Cos-7 cells are reminiscent of ER changes previously reported for airway epithelial cells of mice lacking expression of Best1 (Barro Soria et al., 2009a). OSER can be induced chemically by the drug 1-phenyl-2-decanoyl-amino-3-morpholino-1-propanol hydrochloride (PDMP) (Sprocati et al., 2006). These changes are reversible and are correlated with changes of the intracellular Ca^{2+} homeostasis. We found that OSER in R218C-Best1 expressing Cos-7 cells was paralleled by an increased plateau Ca^{2+}, indicating attenuated recovery from agonist induced increase in $[Ca^{2+}]_i$ (Figure 2D–F). Interestingly, when we used a Ca^{2+}-sensitive GFP probe (GFPCaMP2) that was fused to the C-terminus of Best1 in order to measure intracellular Ca^{2+} in close proximity to the ER, we found that the ATP-induced Ca^{2+} plateau was significantly prolonged (Figure 3). GCaMP2 is an improved bright Ca^{2+} sensor that can be fused to the protein of interest and allows spatial resolution of Ca^{2+} signals in the immediate vicinity of the protein (Nakai et al., 2001; Tallini et al., 2006). Because Best1 is located predominantly in the ER membrane, the data indicate that it controls intracellular Ca^{2+} levels in close proximity to both ER and plasma membrane localized Cl^- channels (Figure 4F).

Best1: upregulated during proliferation, inflammation and in cancer

Both Best1 and CaCCs are upregulated during dedifferentiation and proliferation of epithelial cells, as well as in fast growing cancer cells (Spitzner et al., 2008; Al Dehni et al., 2009). In contrast to proliferating cells, in fully differentiated epithelial cells from human airways, kidney and colon, very little Ca^{2+}-dependent Cl^- conductance is detected (Kunzelmann and Mall, 2002; Al Dehni et al., 2009). Notably, in an island of proliferating M1 cells, Best1 expression is upregulated in edge cells, but is hardly detectable in the center of the island (Figure 5A). Best1 could affect proliferation through its function as a Cl^- channel (Schreiber, 2005) or by controlling intracellular Ca^{2+}-signaling (Barro Soria et al., 2009a). We found that niflumic acid (NFA) inhibition of Ca^{2+}-activated Cl^- channels in M1 cells reduced BrdU incorporation and cell number (Figure 5B). Moreover, NFA treatment induced cellular apoptosis (Figure 5C). As Best1 is upregulated during inflammatory processes and in scratch assays, it could serve as an important regulator of proliferation during tissue repair (Al Dehni et al., 2009). In the

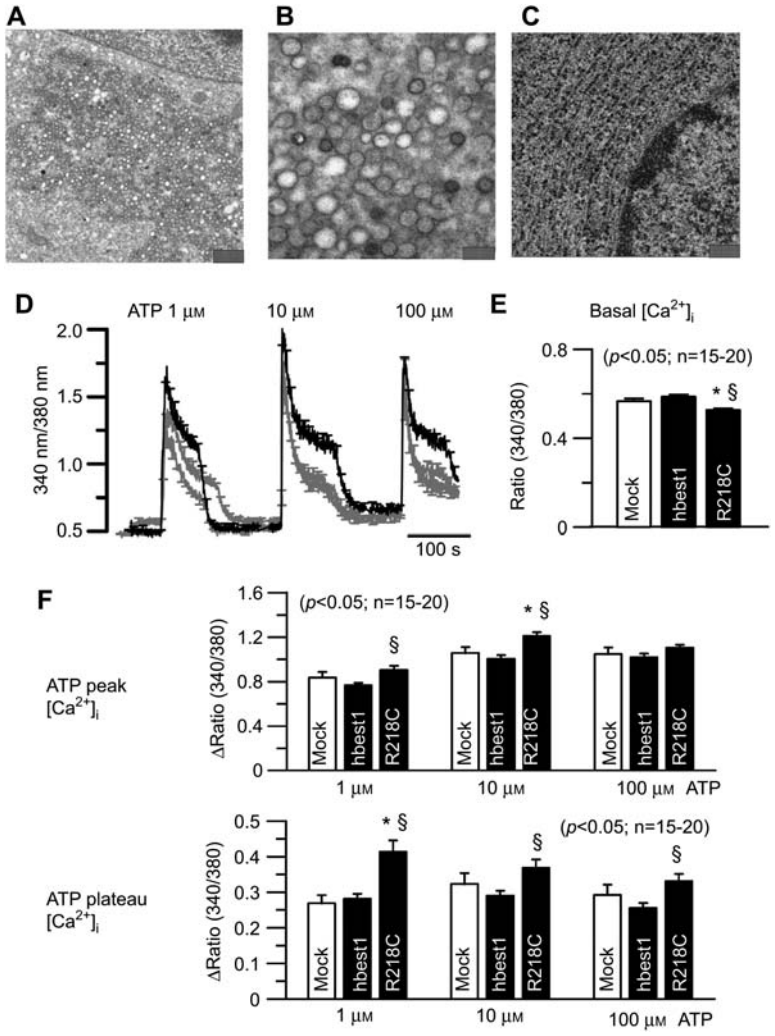

Figure 2 An intracellular function of Best1.
(A, B) Electron microscopy images showing vesicular ER structure in R218C-Best1 expressing Cos-7 cells at lower (bar=500 nm) and higher (bar=200 nm) magnification. (C) Typical tubular ER structure (bar=500 nm). Tetracysteine tagged R218C-Best1-GFP was visualized by binding and photoactivation of the chromophore ReAsH causing DAB precipitation (dark staining). (D) Intracellular Ca^{2+} signals induced by stimulation with three different concentrations of ATP in mock and hBest1 transfected cells (gray curve) and R218C-Best1 expressing Cos-7 cells (black curve). (E, F) Summary of baseline (basal) Ca^{2+} and ATP-induced peak and plateau Ca^{2+} in the different cells (number of cells). * §Statistical significance (analysis of variance, $p<0.05$).

mouse kidney, expression of Best1 was detected in some but not all tubular epithelial cells (Barro Soria et al., 2009b), whereas TMEM16A was detected in renal tubular segments (Figure 5D,E). However, as NFA also inhibits TMEM16A, which is coexpressed together with Best1 in M1 cells and native renal epithelial cells (Yang et al., 2008), the observed effects could be partially due to inhibition of TMEM16A.

Importance of Best1 in epithelial cells

We found that both Best1 and Best2 are expressed in epithelial cells of airways, colon and kidney (Barro Soria et al., 2008, 2009b; Hennig et al., 2008). In the airways, expression of Best2 was found to be much higher when compared with Best1. It was therefore rather surprising that Best1 or Best2 null mice did not reveal any obvious phenotype (Bakall et al., 2008; Barro Soria et al., 2008). Because attenuated Cl^- secretion was detected in Best1 knockout mice, we suggest that Best1 modulates Cl^- secretion rather than being the plasma membrane Cl^- channel (Barro Soria et al., 2008).

Recently a novel function has been proposed for Best2 in the colon epithelium (Yu et al., 2010). It was found that Ca^{2+}-activated anion secretion in the mouse colon was carried mainly by HCO_3^- and was largely reduced in Best2$^{-/-}$ knockout mice. According to this study, Best2 located in the basolateral membrane of goblet cells is obviously in charge of colonic bicarbonate secretion (Yu et al., 2010). It will be interesting to learn whether Best2 is really in charge of basolateral HCO_3^- uptake during Ca^{2+}-dependent secretion or whether it affects HCO_3^- secretion by controlling intracellular Ca^{2+} signals.

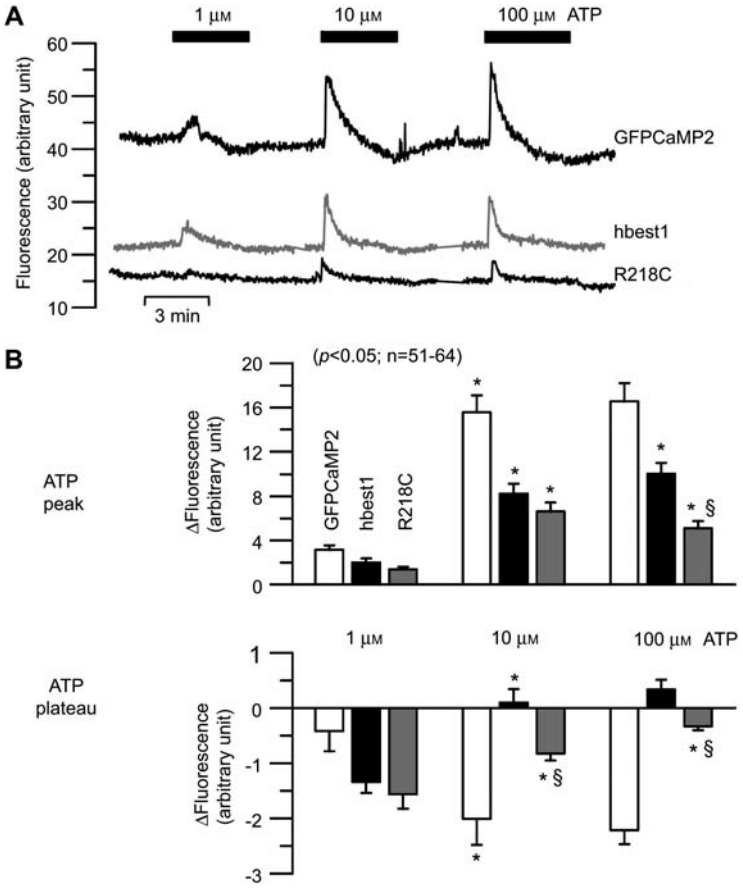

Figure 3 Best1 changes Ca^{2+} signals in close proximity to the ER and membrane channels.
(A) Original recordings of fluorescence signals measured in Cos-7 cells, which had been transfected with Ca^{2+} sensitive GFP (GFPCaMP2) alone or as C-terminal fusion protein with hbest1 (hbest1) or hbest1-R218C (R218C) to measure intracellular Ca^{2+} changes close to the ER membrane and in close proximity to Best1. In the presence of ATP, Ca^{2+} concentrations sensed by GFPCaMP2 increased transiently (ATP peak) followed by a reduction in the Ca^{2+} concentration below baseline levels (ATP plateau, negative changes in B). This could be due to activation of Ca^{2+} uptake back into ER stores. Overexpression of hbest1 but not of the pore mutant hbest1-R218C enhanced the Ca^{2+} plateau induced by ATP stimulation (positive changes, see summary). (B) Summary of ATP-induced peak and plateau Ca^{2+} (number of cells). *Statistical significance (paired t-test), §statistical significance to hbest1 (unpaired t-test; $p<0.05$).

TMEM16A is a major component of the long awaited Ca^{2+}-activated Cl^- channel

TMEM16A is the Ca^{2+}-activated Cl^- channel or at least a major component of it that shares most of the properties that have been ascribed to endogenous CaCCs, whereas other biophysical properties such as the single channel conductance was found to be different to endogenous channels (Yang et al., 2008). Whereas Yang and colleagues searched public domain databases for putative channel genes (Yang et al., 2008), Schroeder and coworkers expression cloned TMEM16A in Axolotl oocytes, which lack endogenous CaCCs (Schroeder et al., 2008). Caputo et al. found TMEM16A by analyzing genes that are induced by interleukin 4, which also induce Ca^{2+}-activated Cl^- conductance (Caputo et al., 2008). TMEM16A belongs to a family of 10 TMEM16 proteins (TMEM16A–K, ANO 1–10). TMEM16A has eight putative transmembrane domains and a p-loop between transmembrane domains 5 and 6. A putative pore forming region was suggested from experiments with side-directed mutagenized channels. TMEM16 proteins have no obvious similarity to other ion channels (Galietta, 2009; Kunzelmann et al., 2009). Moreover, TMEM16 proteins exist as multiple alternatively spliced products in different cell types (Caputo et al., 2008).

Epithelial relevance of TMEM16A channels

An increasing number of studies demonstrate the relevance of TMEM16A in epithelial tissues. The comprehensive analysis of Yang et al. indicated predominant expression in epithelial tissues (Yang et al., 2008). Expression in both mammary and salivary glands was also shown by *in situ* hybridization and a function of TMEM16A in salivary glands has been subsequently demonstrated (Schroeder et al., 2008; Ousingsawat et al., 2009; Romanenko et al., 2010). We analyzed the role of TMEM16A in a broad spectrum of epithelial tissues, including airways, colonic epithelium, pancreatic acinar cells, salivary gland cells and hepatocytes

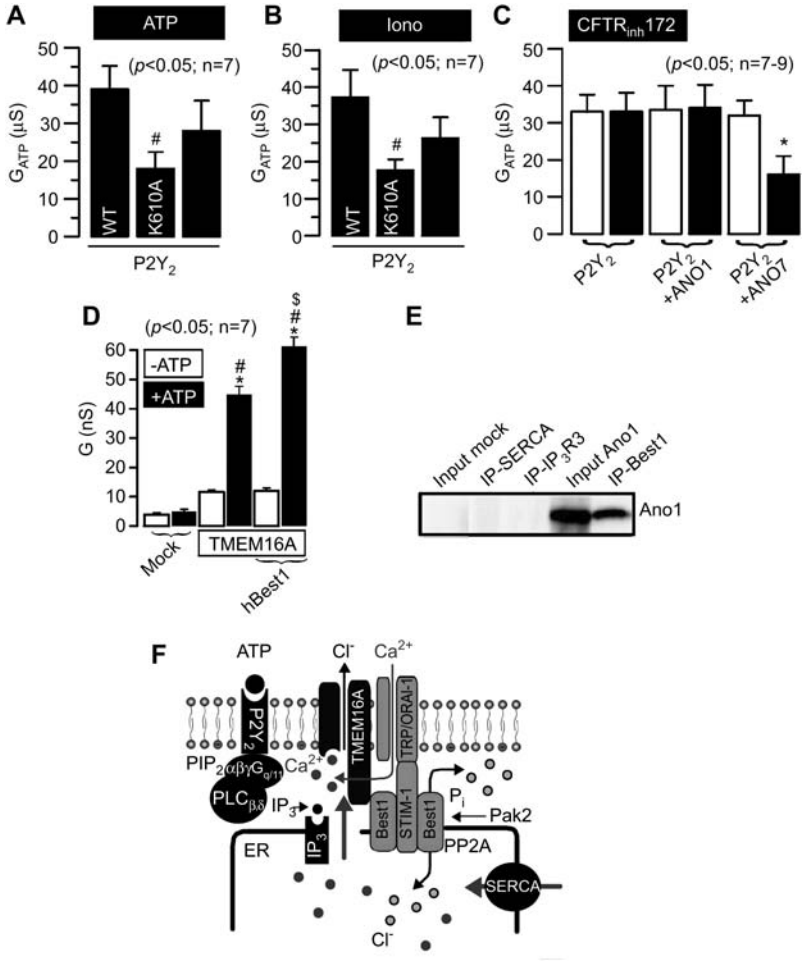

Figure 4 TMEM16 channels work in concert with other proteins.
(A, B) Whole cell conductances in *Xenopus* oocytes coexpressing $P2Y_2$ receptors and human wild-type TMEM16A, K610A–TMEM16A or $P2Y_2$ receptors only. Whole cell conductances were activated by ATP (100 µM) or ionomycin (1 µM). (C) Whole cell conductances in *Xenopus* oocytes expressing $P2Y_2$ receptors only or coexpressing $P2Y_2$ receptors and human TMEM16A or TMEM16G, and effect of $CFTR_{inh}172$. (D) Summary of whole cell conductances measured in mock transfected HEK293 cells and in cells expressing TMEM16A or coexpressing TMEM16A and Best1 (number of cells). *Statistical significance (paired t-test; $p<0.05$), #statistical significance (unpaired t-test; $p<0.05$). (E) Best1 but not SERCA or IP3R3 was immunoprecipitated with TMEM16A. Best1 was coexpressed with TMEM16A, SERCA or IP3R3 in HEK293 cells. TMEM16A was coimmunoprecipitated from cell lysates using a Best1 antibody (from Davids Technologie, Regensburg, Germany). The TMEM16A antibody was from Davids Technologie; all other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). (F) Model for a hypothetical functional microdomain at the ER/plasma membrane interface that contains ion channels and transporters in charge of Ca^{2+}-signaling at Cl^- secretion. $ Statistical significance to TMEM16A expressing cells stimulated with ATP (unpaired t-test; $p<0.05$).

(Ousingsawat et al., 2009). In all of the tissues we found that TMEM16A has a major contribution to Ca^{2+}-activated whole cell currents and to Ca^{2+}-dependent Cl^- secretion, respectively. These experiments became possible because of a TMEM16A-knockout mouse model that had been generated earlier (Rock et al., 2008). Assessment of epithelial function in these animals has been difficult, because neonatal animals show a reduced weight and because they die within a couple of days after birth (Rock et al., 2008).

Loss of TMEM16A function leads to multiple problems in epithelial organs. Rock and colleagues found accumulation of mucous in the lumen of tracheas of TMEM16A null mice, whereas we detected missing cholinergic activation of mucociliary clearance in tracheas of TMEM16A null mice (Ousingsawat et al., 2009; Rock et al., 2009). This is supported by the finding of Lee and Foskett who found TMEM16A in porcine bronchial submucosal gland serous acinar cells (Lee and Foskett, 2009). The work by Yang et al. and Romanenko et al. demonstrates the importance of TMEM16A for saliva production (Yang et al., 2008; Romanenko et al., 2010). Recent studies propose TMEM16A as an intestinal secretory channel during diarrhea that could be pharmacologically targeted (Tradtrantip et al., 2009). This could be particularly important during infectious rotavirus-induced diarrhea (Ousingsawat et al., submitted to Europ. J. Physiol. 2010). Conditional $TMEM16A^{-/-}$ mice are currently

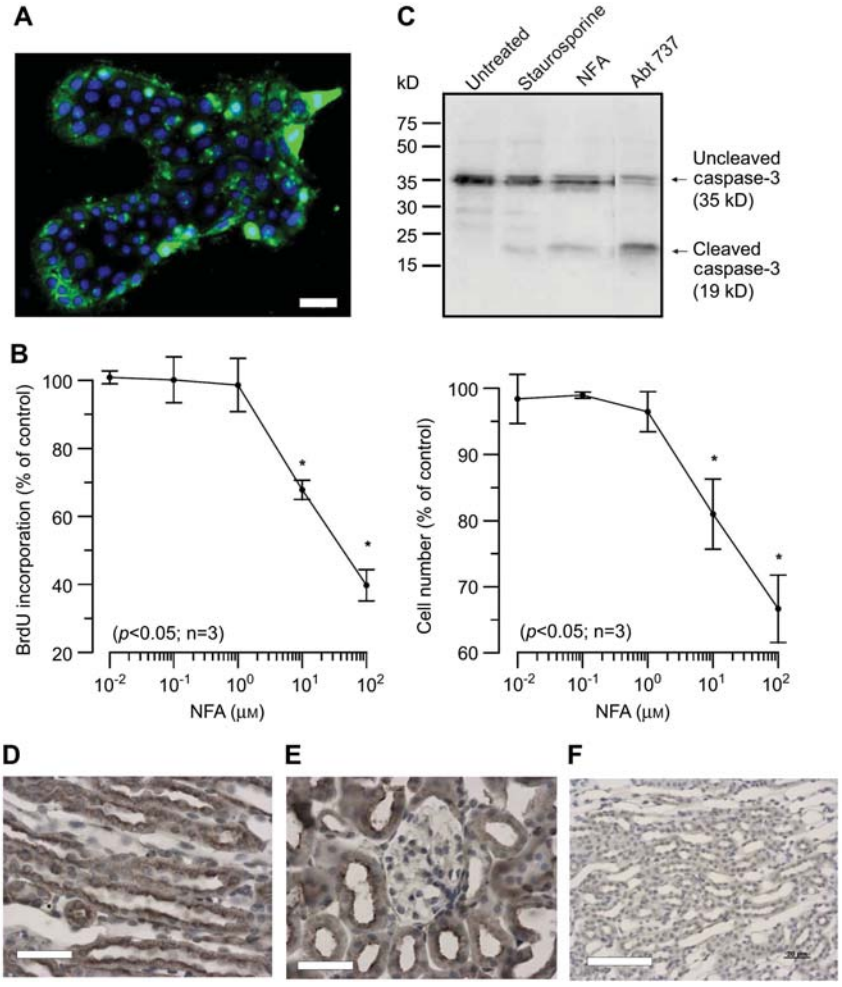

Figure 5 Best1: upregulated during proliferation, inflammation and in cancer.
(A) Immunocytochemistry of Best1 expressed in the mouse cortical collecting duct cell line M1. Best1 is clearly expressed in proliferating cells at the edges of cell islands, whereas non-proliferating cells in the center of the monolayers do not show expression of Best1 (bar=20 μm). (B) Inhibition of BrdU incorporation and cell number by different concentrations of niflumic acid (NFA). Western blot analysis of uncleaved and cleaved caspase-3. Cells have been grown in the presence of Staurosporine (0.5 μM), NFA (at the concentrations indicated) and the BCL-2 inhibitor Abt 737 (20 mM) for 24 h (number of cells). *Statistical significance (unpaired t-test; $p<0.05$). (D, E) Immunohistochemistry of TMEM16A in mouse kidney (bar=30 μm), and (F) control (secondary antibody only; bar=50 μm). Anti-TMEM16A antibodies were from Davids Biotechnologie.

generated and will be needed to fully evaluate the role of TMEM16A in other tissues such as intestine, kidney, liver and pancreas.

Other exciting functions of TMEM16A

As outlined above, expression of CaCCs is abundant and for TMEM16A and the other members of the TMEM16 family a rather broad expression has been demonstrated (Schreiber et al., 2010). It therefore does not come as a surprise that evermore functions of TMEM16 proteins are identified. In pulmonary arterial smooth muscle cells, TMEM16A mediates CaCCs, which supports contraction and which could therefore become a major pharmacological target in the near future (Tradtrantip et al., 2009; Manoury et al., 2010). TMEM16A expressed in interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles and thus for intestinal motility (Gomez-Pinilla et al., 2009; Huang et al., 2009; Hwang et al., 2009). Other reports strengthen the impact of TMEM16A on neuronal function and show that TMEM16A is important for production of nociceptive signals (Brown and Passmore, 2010; Liu et al., 2010).

Recent research also shows a role of TMEM16A in erythrocytes. We demonstrated that TMEM16A contributes substantially to hemolysin-induced cell volume reduction because erythrocytes isolated from TMEM16A$^{-/-}$ mice show significantly attenuated crenation and increased lysis compared with controls (Skals et al., 2010). These findings also imply a role of TMEM16A in cell volume regulation. All vertebrate cells regulate their cell volume by activating K^+

and Cl^- channels and releasing ion to reduce intracellular osmotic strength. The responsible volume-regulated Cl^- channels ($I_{Cl,swell}$) have not been identified. Volume- and Ca^{2+}-regulated channels have distinct properties but share common features such as pharmacological features, outward rectification or anion selectivity. We show that TMEM16A together with other TMEM16 proteins are activated by cell swelling through an autocrine mechanism that involves ATP release and binding to purinergic $P2Y_2$ receptors. It is obvious that TMEM16A currents produce a time-dependent activation of whole cell Cl^- currents, whereas the typical volume-regulated Cl^- current ($I_{Cl,swell}$) shows a time-dependent inactivation (Hoffmann et al., 2009). However, our present data indicate that TMEM16A in conjunction with other TMEM16 members produce $I_{Cl,swell}$, and it is not yet clear how the TMEM16 paralogs affect the properties of the TMEM16 current (Almaca et al., 2009). Notably, also Best1 is regulated by volume, although this regulatory pathway remains to be demonstrated in human cells (Fischmeister and Hartzell, 2005).

TMEM16A channels are activated by ATP through increase in intracellular Ca^{2+} and by a Ca^{2+}-independent mechanism engaging extracellular regulated protein kinases (Erk1/2) (Almaca et al., 2009). We demonstrate a role of TMEM16A for $I_{Cl,swell}$ and regulatory volume decrease in recombinant cells, cells expressing TMEM16 proteins endogenously and in epithelial tissues of isolated mice lacking expression of TMEM16A. These results demonstrate a crucial role of TMEM16 proteins for volume-regulated Cl^- channels, which could therefore be important for proliferation and apoptotic cell death (Almaca et al., 2009). Finally, for other TMEM16 proteins physiological functions have also been reported meanwhile: TMEM16B provides a ciliary pathway for excitatory Cl^- currents in mammalian olfactory cells (Stephan et al., 2009; Hengl et al., 2010) and also forms a presynaptic protein complex in photoreceptor terminals of the retina (Stohr et al., 2009). In contrast, TMEM16G could be the important CaCC in primate taste receptors (Moyer et al., 2009).

TMEM16 channels work in concert with other proteins

Regulation of TMEM16 appears complex and difficult to determine. A recent analysis of all TMEM16 proteins (16A–16K, ANO1–ANO10) indicated a broad expression in all mammalian tissues and in different combinations (Schreiber et al., 2010). Epithelial cells express mainly ANO1, 6, 7, 8, 9, 10, whereas neuronal and muscle cells express ANO2, 3, 4, 5. Every cell type expresses several TMEM16 proteins and most cell lines we looked at come with a standard equipment of 16F, 16H, 16J together with more variable expression of the other TMEM16 proteins. Even *Xenopus* oocytes express several of these channels, such as ANO1, ANO5 and ANO10 (data not shown). Such background activity compromises the analysis of overexpressed TMEM16 channels. Most TMEM16 proteins are membrane localized, whereas TMEM16H, J, K are located in the cytosol (Schreiber et al., 2010). Thus, most TMEM16 channels do not reliably produce CaCCs, and TMEM16J and TMEM16K were even inhibitory on other TMEM16 proteins (Schreiber et al., 2010). It seems that TMEM16 proteins hetero-oligomerize and can therefore form Cl^- channels with particular properties in individual tissues (Almaca et al., 2009). When the pore mutant hTMEM16A–K610A was expressed in *Xenopus* oocytes, the endogenous CaCC activated by stimulation of purinergic receptors with ATP or by ionomycin was suppressed (Figure 4A,B), which suggests that the mutant protein interferes with wild-type protein by hetero-oligomerization. This probably also takes place in native mammalian cells because both Best1 and TMEM16A are coexpressed in the same epithelial cells (Kunzelmann et al., 2009; Ousingsawat et al., 2009; Barro Soria et al., 2009b). Coexpression of TMEM16 proteins can also change pharmacological properties, which could explain why CaCCs in different tissues have variable pharmacological properties (Hartzell et al., 2005a). Interestingly we found that expression of TMEM16G, which has been shown to produce a CaCC (Schreiber et al., 2010), did not enhance the magnitude of CaCCs in *Xenopus* oocytes, but made it sensitive for inhibition by 20 μM $CFTR_{inh}172$, a channel blocker that typically only inhibits CFTR (Figure 4C) (Ma et al., 2002). TMEM16 proteins might not only interact with themselves but also with other proteins regulating channel activity (Barro Soria et al., 2009a; Forrest et al., 2010). Excitingly, we were able to coimmunoprecipitate Best1 with TMEM16A upon coexpression of both proteins in HEK293 cells (Figure 4D,E). This interaction probably also takes place in native tissues because both TMEM16A and Best1 are coexpressed in airway, colonic and renal epithelial cells (Al Dehni et al., 2009; Kunzelmann et al., 2009; Barro Soria et al., 2009b). To fully understand the function and regulation of CaCCs and TMEM16 proteins in particular, it will be necessary to appreciate all of these molecular relationships and to gain more insight into these functional microdomains (Figure 4F). It will also be exciting to learn how the different TMEM16 paralogs operate together to produce Cl^- currents in a cell-specific manner to meet the requirements of an individual tissue.

Acknowledgments

Our research is continuously supported by the Deutsche Forschungsgemeinschaft SFB699/A7 and TargetScreen2 (EU-FP6-2005-LH-037365). We thank Christine Meese und Karin Schadendorf for their excellent technical assistance.

References

Al Dehni, F., Spitzner, M., Martins, J.R., Barro Soria, R., Schreiber, R., and Kunzelmann, K. (2009). Role of bestrophin for proliferation and in epithelial to mesenchymal transition. J. Am. Soc. Nephrol. *20*, 1556–1564.

Almaca, J., Tian, Y., Al Dehni, F., Ousingsawat, J., Kongsuphol, P., Rock, J.R., Harfe, B.D., Schreiber, R., and Kunzelmann, K. (2009). TMEM16 proteins produce volume regulated chloride

currents that are reduced in mice lacking TMEM16A. J. Biol. Chem. *284*, 28571–28578.

Bakall, B., McLaughlin, P., Stanton, J.B., Zhang, Y., Hartzell, H.C., Marmorstein, L.Y., and Marmorstein, A.D. (2008). Bestrophin-2 is involved in the generation of intraocular pressure. Invest. Ophthalmol. Vis. Sci. *49*, 1563–1570.

Barro Soria, R., Schreiber, R., and Kunzelmann, K. (2008). Bestrophin 1 and 2 are components of the Ca^{2+} activated Cl^- conductance in mouse airways. Biochim. Biophys. Acta *1783*, 1993–2000.

Barro Soria, R., Al Dehni, F., Almaca, J., Witzgall, R., Schreiber, R., and Kunzelmann, K. (2009a). ER localized bestrophin1 acts as a counter-ion channel to activate Ca^{2+} dependent ion channels TMEM16A and SK4. Pflüger's Arch. *459*, 485–497.

Barro Soria, R., Spitzner, M., Schreiber, R., and Kunzelmann, K. (2009b). Bestrophin 1 enables Ca^{2+} activated Cl^- conductance in epithelia. J. Biol. Chem. *284*, 29405–29412.

Borgese, N., Francolini, M., and Snapp, E. (2006). Endoplasmic reticulum architecture: structures in flux. Curr. Opin. Cell Biol. *18*, 358–364.

Brown, D.A. and Passmore, G.M. (2010). Some new insights into the molecular mechanisms of pain perception. J. Clin. Invest. *120*, 1380–1383.

Caputo, A., Caci, E., Ferrera, L., Pedemonte, N., Barsanti, C., Sondo, E., Pfeffer, U., Ravazzolo, R., Zegarra-Moran, O., and Galietta, L.J. (2008). TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science *322*, 590–594.

Duran, C., Thompson, C.H., Xiao, Q., and Hartzell, H.C. (2010). Chloride channels: often enigmatic, rarely predictable. Annu. Rev. Physiol. *17*, 95–121.

Eggermont, J. (2004). Calcium-activated chloride channels: (un)known, (un)loved? Proc. Am. Thorac. Soc. *1*, 22–27.

Fischmeister, R. and Hartzell, H.C. (2005). Volume sensitivity of the bestrophin family of chloride channels. J. Physiol. *562*, 477–491.

Forrest, A.S., Angermann, J.E., Raghunathan, R., Lachendro, C., Greenwood, I.A., and Leblanc, N. (2010). Intricate interaction between store-operated calcium entry and calcium-activated chloride channels in pulmonary artery smooth muscle cells. Adv. Exp. Med. Biol. *661*, 31–55.

Frings, S., Reuter, D., and Kleene, S.J. (2000). Neuronal Ca^{2+}-activated Cl^- channels – homing in on an elusive channel species. Prog. Neurobiol. *60*, 247–289.

Galietta, L.J. (2009). The TMEM16 protein family: a new class of chloride channels? Biophys. J. *97*, 3047–3053.

Gomez-Pinilla, P.J., Gibbons, S.J., Bardsley, M.R., Lorincz, A., Pozo, M.J., Pasricha, P.J., van de Rijn, M., West, R.B., Sarr, M.G., Kendrick, M.L., et al. (2009). Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. Am. J. Physiol. Gastrointest. Liver Physiol. *296*, G1370–G1381.

Griffin, B.A., Adams, S.R., and Tsien, R.Y. (1998). Specific covalent labeling of recombinant protein molecules inside live cells. Science *281*, 269–272.

Hartzell, H.C. (2008). Molecular physiology of bestrophins: multifunctional membrane proteins linked to Best disease and other retinopathies. Physiol. Rev. *88*, 639–672.

Hartzell, H.C., Putzier, I., and Arreola, J. (2005a). Calcium-activated chloride channels. Annu. Rev. Physiol. *67*, 719–758.

Hartzell, H.C., Qu, Z., Putzier, I., Artinian, L., Chien, L.T., and Cui, Y. (2005b). Looking chloride channels straight in the eye: bestrophins, lipofuscinosis, and retinal degeneration. Physiology (Bethesda) *20*, 292–302.

Hartzell, H.C., Yu, K., Xiao, Q., Chien, L.T., and Qu, Z. (2008). Anoctamin/TMEM16 family members are Ca^{2+}-activated Cl^- channels. J. Physiol. *587*, 2127–2139.

Hengl, T., Kaneko, H., Dauner, K., Vocke, K., Frings, S., and Mohrlen, F. (2010). Molecular components of signal amplification in olfactory sensory cilia. Proc. Natl. Acad. Sci. USA *107*, 6052–6057.

Hennig, B., Schultheiss, G., Kunzelmann, K., and Diener, M. (2008). Ca^{2+}-Induced Cl^- efflux at rat distal colonic epithelium. J. Membr. Biol. *221*, 61–72.

Hoffmann, E.K., Lambert, I.H., and Pedersen, S.F. (2009). Physiology of cell volume regulation in vertebrates. Physiol. Rev. *89*, 193–277.

Huang, F., Rock, J.R., Harfe, B.D., Cheng, T., Huang, X., Jan, Y.N., and Jan, L.Y. (2009). Studies on expression and function of the TMEM16A calcium-activated chloride channel. Proc. Natl. Acad. Sci. USA *106*, 21413–21418.

Hwang, S.J., Blair, P.J., Britton, F.C., O'Driscoll, K.E., Hennig, G., Bayguinov, Y.R., Rock, J.R., Harfe, B.D., Sanders, K.M., and Ward, S.M. (2009). Expression of anoctamin 1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. J. Physiol. *587*, 4887–4904.

Kidd, J.F. and Thorn, P. (2000). Intracellular Ca^{2+} and Cl^- channel activation in secretory cells. Annu. Rev. Physiol. *62*, 493–513.

Kunzelmann, K. and Mall, M. (2002). Electrolyte transport in the colon: mechanisms and implications for disease. Physiol. Rev. *82*, 245–289.

Kunzelmann, K., Kongsuphol, P., Al Dehni, F., Tian, Y., Ousingsawat, J., Warth, R., and Schreiber, R. (2009). Bestrophin and TMEM16 – Ca^{2+} activated Cl^- channels with different functions. Cell Calcium *46*, 233–241.

Leblanc, N., Ledoux, J., Saleh, S., Sanguinetti, A., Angermann, J., O'Driscoll, K.E., Britton, F., Perrino, B.A., and Greenwood, I.A. (2005). Regulation of calcium-activated chloride channels in smooth muscle cells: a complex picture is emerging. Can. J. Physiol. Pharmacol. *83*, 541–556.

Lee, R.J. and Foskett, J.K. (2009). Mechanisms of Ca^{2+}-stimulated fluid secretion by porcine bronchial submucosal gland serous acinar cells. Am. J. Physiol. Lung Cell Mol. Physiol. *298*, L210–L231.

Liu, B., Linley, J.E., Du, X., Zhang, X., Ooi, L., Zhang, H., and Gamper, N. (2010). The acute nociceptive signals induced by bradykinin in rat sensory neurons are mediated by inhibition of M-type K^+ channels and activation of Ca^{2+}-activated Cl^- channels. J. Clin. Invest. *120*, 1240–1252.

Ma, T., Thiagarajah, J.R., Yang, H., Sonawane, N.D., Folli, C., Galietta, L.J., and Verkman, A.S. (2002). Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. J. Clin. Invest. *110*, 1651–1658.

Manoury, B., Tamuleviciute, A., and Tammaro, P. (2010). TMEM16A/Anoctamin1 protein mediates calcium-activated chloride currents in pulmonary arterial smooth muscle cells. J. Physiol. *588*, 2305–2314.

Marmorstein, A.D. and Kinnick, T.R. (2007). Focus on molecules: Bestrophin (Best-1). Exp. Eye Res. *85*, 423–424.

Marmorstein, A.D., Marmorstein, L.Y., Rayborn, M., Wang, X., Hollyfield, J.G., and Petrukhin, K. (2000). Bestrophin, the product of the Best vitelliform macular dystrophy gene (VMD2), localizes to the basolateral plasma membrane of the retinal pigment epithelium. Proc. Natl. Acad. Sci. USA *97*, 12758–12763.

Marmorstein, L.Y., Wu, J., McLaughlin, P., Yocom, J., Karl, M.O., Neussert, R., Wimmers, S., Stanton, J.B., Gregg, R.G., Strauss, O., et al. (2006). The light peak of the electroretinogram is

dependent on voltage-gated calcium channels and antagonized by Bestrophin (Best-1). J. Gen. Physiol. *127*, 577–589.

Matchkov, V.V., Larsen, P., Bouzinova, E.V., Rojek, A., Briggs Boedtkjer, D.M., Golubinskaya, V., Pedersen, F.S., Aalkjaer, C., and Nilsson, H. (2008). Bestrophin-3 (vitelliform macular dystrophy 2-like 3 protein) is essential for the cGMP-dependent calcium-activated chloride conductance in vascular smooth muscle cells. Circ. Res. *103*, 864–872.

Melvin, J.E., Yule, D., Shuttleworth, T., and Begenisich, T. (2005). Regulation of fluid and electrolyte secretion in salivary gland acinar cells. Annu. Rev. Physiol. *67*, 445–469.

Moyer, B.D., Hevezi, P., Gao, N., Lu, M., Kalabat, D., Soto, H., Echeverri, F., Laita, B., Yeh, S.A., Zoller, M., et al. (2009). Expression of genes encoding multi-transmembrane proteins in specific primate taste cell populations. PLoS ONE *4*, e7682.

Nakai, J., Ohkura, M., and Imoto, K. (2001). A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. Nat. Biotechnol. *19*, 137–141.

Neussert, R., Muller, C., Milenkovic, V.M., and Strauss, O. (2010). The presence of bestrophin-1 modulates the Ca^{2+} recruitment from Ca^{2+} stores in the ER. Pflüger's Arch. *460*, 163–175.

O'Driscoll, K.E., Hatton, W.J., Burkin, H.R., Leblanc, N., and Britton, F.C. (2008). Expression, localization, and functional properties of Bestrophin 3 channel isolated from mouse heart. Am. J. Physiol. Cell Physiol. *295*, C1610–C1624.

O'Driscoll, K.E., Leblanc, N., Hatton, W.J., and Britton, F.C. (2009). Functional properties of murine bestrophin 1 channel. Biochem. Biophys. Res. Commun. *384*, 476–481.

Ousingsawat, J., Martins, J.R., Schreiber, R., Rock, J.R., Harfe, B.D., and Kunzelmann, K. (2009). Loss of TMEM16A causes a defect in epithelial Ca^{2+} dependent chloride transport. J. Biol. Chem. *284*, 28698–28703.

Petrukhin, K., Koisti, M.J., Bakall, B., Li, W., Xie, G., Marknell, T., Sandgren, O., Forsman, K., Holmgren, G., Andreasson, S., et al. (1998). Identification of the gene responsible for Best macular dystrophy. Nat. Genet. *19*, 241–247.

Qu, Z. and Hartzell, H.C. (2004). Determinants of anion permeation in the second transmembrane domain of the mouse bestrophin-2 chloride channel. J. Gen. Physiol. *124*, 371–382.

Qu, Z., Wei, R.W., Mann, W., and Hartzell, H.C. (2003). Two bestrophins cloned from *Xenopus laevis* oocytes express Ca^{2+}-activated Cl^- currents. J. Biol. Chem. *278*, 49563–49572.

Rock, J.R., Futtner, C.R., and Harfe, B.D. (2008). The transmembrane protein TMEM16A is required for normal development of the murine trachea. Dev. Biol. *321*, 141–149.

Rock, J.R., O'Neal, W.K., Gabriel, S.E., Randell, S.H., Harfe, B.D., Boucher, R.C., and Grubb, B.R. (2009). Transmembrane protein 16A (TMEM16A) is a Ca^{2+} regulated Cl^--secretory channel in mouse airways. J. Biol. Chem. *284*, 14875–14880.

Romanenko, V.G., Catalan, M.A., Brown, D.A., Putzier, I., Hartzell, H.C., Marmorstein, A.D., Gonzalez-Begne, M., Rock, J.R., Harfe, B.D., and Melvin, J.E. (2010). Tmem16A encodes the Ca^{2+}-activated Cl^- channel in mouse submandibular salivary gland acinar cells. J. Biol. Chem. *285*, 12990–13001.

Rosenthal, R., Bakall, B., Kinnick, T., Peachey, N., Wimmers, S., Wadelius, C., Marmorstein, A.D., and Strauss, O. (2006). Expression of bestrophin-1, the product of the VMD2 gene, modulates voltage-dependent Ca^{2+} channels in retinal pigment epithelial cells. FASEB J. *20*, 178–180.

Schreiber, R. (2005). Ca^{2+} signaling, intracellular pH and cell volume in cell proliferation. J. Membr. Biol. *205*, 129–137.

Schreiber, R., Uliyakina, I., Kongsuphol, P., Warth, R., Mirza, M., Martins, J.R., and Kunzelmann, K. (2010). Expression and function of epithelial anoctamins. J. Biol. Chem. *285*, 7838–7845.

Schroeder, B.C., Cheng, T., Jan, Y.N., and Jan, L.Y. (2008). Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell *134*, 1019–1029.

Skals, M., Jensen, U.B., Ousingsawat, J., Kunzelmann, K., Leipziger, J., and Praetorius, H.A. (2010). *E. coli* α-hemolysin triggers shrinkage of erythrocytes via KCa3.1 and TMEM16A channels with subsequent phosphatidyl serine exposure. J. Biol. Chem. *285*, 15557–15565.

Spitzner, M., Martins, J.R., Barro Soria, R., Ousingsawat, J., Scheidt, K., Schreiber, R., and Kunzelmann, K. (2008). Eag1 and Bestrophin 1 are upregulated in fast growing colonic cancer cells. J. Biol. Chem. *283*, 7421–7428.

Sprocati, T., Ronchi, P., Raimondi, A., Francolini, M., and Borgese, N. (2006). Dynamic and reversible restructuring of the ER induced by PDMP in cultured cells. J. Cell Sci. *119*, 3249–3260.

Stephan, A.B., Shum, E.Y., Hirsh, S., Cygnar, K.D., Reisert, J., and Zhao, H. (2009). ANO2 is the cilial calcium-activated chloride channel that may mediate olfactory amplification. Proc. Natl. Acad. Sci. USA *106*, 11776–11781.

Stohr, H., Heisig, J.B., Benz, P.M., Schoberl, S., Milenkovic, V.M., Strauss, O., Aartsen, W.M., Wijnholds, J., Weber, B.H., and Schulz, H.L. (2009). TMEM16B, a novel protein with calcium-dependent chloride channel activity, associates with a presynaptic protein complex in photoreceptor terminals. J. Neurosci. *29*, 6809–6818.

Sun, H., Tsunenari, T., Yau, K.W., and Nathans, J. (2001). The vitelliform macular dystrophy protein defines a new family of chloride channels. Proc. Natl. Acad. Sci. USA *99*, 4008–4013.

Tallini, Y.N., Ohkura, M., Choi, B.R., Ji, G., Imoto, K., Doran, R., Lee, J., Plan, P., Wilson, J., Xin, H.B., et al. (2006). Imaging cellular signals in the heart *in vivo*: cardiac expression of the high-signal Ca^{2+} indicator GCaMP2. Proc. Natl. Acad. Sci. USA *103*, 4753–4758.

Tradtrantip, L., Namkung, W., and Verkman, A.S. (2009). Crofelemer, an antisecretory antidiarrheal proanthocyanidin oligomer extracted from *Croton lechleri*, targets two distinct intestinal chloride channels. Mol. Pharmacol. *77*, 69–78.

Tsunenari, T., Sun, H., Williams, J., Cahill, H., Smallwood, P., Yau, K.W., and Nathans, J. (2003). Structure-function analysis of the bestrophin family of anion channels. J. Biol. Chem. *278*, 41114–41125.

Xiao, Q., Hartzell, H.C., and Yu, K. (2010). Bestrophins and retinopathies. Pflüger's Arch. *460*, 559–569.

Yang, Y.D., Cho, H., Koo, J.Y., Tak, M.H., Cho, Y., Shim, W.S., Park, S.P., Lee, J., Lee, B., Kim, B.M., et al. (2008). TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature *455*, 1210–1215.

Yu, K., Cui, Y., and Hartzell, H.C. (2006). The bestrophin mutation A243V, linked to adult-onset vitelliform macular dystrophy, impairs its chloride channel function. Invest. Ophthalmol. Vis. Sci. *47*, 4956–4961.

Yu, K., Xiao, Q., Cui, G., Lee, A., and Hartzell, H.C. (2008). The best disease-linked Cl^- channel hBest1 regulates Ca V 1 (L-type) Ca^{2+} channels via src-homology-binding domains. J. Neurosci. *28*, 5660–5670.

Yu, K., Lujan, R., Marmorstein, A., Gabriel, S.E., and Hartzell, H.C. (2010). Bestrophin-2 mediates bicarbonate transport by goblet cells in mouse colon. J. Clin. Invest. *120*, 1722–1735.

Zhang, Y., Stanton, J.B., Wu, J., Yu, K., Hartzell, H.C., Peachey, N.S., Marmorstein, L.Y., and Marmorstein, A.D. (2010). Suppression of Ca^{2+} signaling in a mouse model of Best disease. Hum. Mol. Genet. *19*, 1108–1118.

Received August 16, 2010; accepted October 11, 2010