Expression and function of epithelial anoctamins

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Calcium-activated Cl\(^-\) currents (CaCCs) are abundant and present in nearly every cell type (Hartzell et al. 2005). Calcium-activated Cl\(^-\) currents fulfil very different functions in excitable and non-excitable tissues, which have been well described in several recent reviews (Hartzell et al. 2005; Melvin et al. 2005; Kunzelmann et al. 2011b). Although other Ca\(^{2+}\)-activated Cl\(^-\) channels have been reported, such as the bestrophins, it is now well accepted that anoctamins (TMEM16 proteins) form plasma-membrane-localized Ca\(^{2+}\)-activated Cl\(^-\) channels (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008). As molecular characteristics and properties of anoctamin 1 (Ano1) have been reported earlier, we will focus on some interesting aspects of overexpressed Ano1 and present evidence for a Cl\(^-\) channel function of anoctamin 6 (Ano6; Hartzell et al. 2005; Melvin et al. 2005; Kunzelmann et al. 2011b).

**Lessons from the anoctamin knockout mouse models**

Calcium-activated Cl\(^-\) current has a role in most tissues and thus anoctamins, particularly Ano1, are found in a wide range of tissues (Kunzelmann et al. 2011b). Expression of anoctamins has been analysed primarily in mice (Rock et al. 2008, 2009; Schroeder et al. 2008; Yang et al. 2008; Huang et al. 2009; Ousingsawat et al. 2009; Romanenko et al. 2010; Schreiber et al. 2010), and fairly little is known about expression in native human tissues. It should be emphasized that in contrast to the dominating role of cystic fibrosis transmembrane conductance regulator (CFTR) in human epithelial transport, mouse physiology is very much based on CaCC (Kunzelmann et al. 2009). The mouse demonstrates broad Ano1 expression and generally pronounced Ca\(^{2+}\)-activated Cl\(^-\) currents (Kunzelmann et al. 2009; Schreiber et al. 2010). It was therefore not surprising to find that mice lacking Ano1 are severely ill, and suffer from numerous transport defects in a wide range of epithelial organs (Rock et al. 2008, 2009; Almac\'\'a et al. 2009; Ousingsawat et al. 2009; Huang et al. 2009; Romanenko et al. 2010).

Pre- and postnatal development of Ano1 null mice is severely compromised, and animals die soon after birth, probably due to malnutrition and instability of the airways (Rock et al. 2008; Huang et al. 2009; Ousingsawat et al. 2009). Obviously, Ano1 is important for proper function of both intestinal and airway pacemaker and smooth
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muscle cells, and lack of Ano1 results in reduced smooth muscle contraction, leading to impaired digestion and developmental malformation of tracheal cartilage rings (Rock et al. 2008; Huang et al. 2009; Fig. 1). We found earlier that Ca$^{2+}$-mediated Cl$^-$ secretion stimulated by carbachol is largely impaired in Ano1 knockout animals (Ousingsawat et al. 2009), and this loss of Cl$^-$ secretion is paralleled by reduced airway contraction (Fig. 1B). Notably, a similar congenital tracheal malformation was found in CFTR-deficient mice (Bonvin et al. 2008). This is probably due to the fact that both CFTR and Ano1 have a role in airway smooth muscle contraction and the generation of mechanical forces necessary for prenatal formation of tracheal cartilage rings (Rock et al. 2008).

Interestingly, loss of anoctamin expression, leading to loss of Ca$^{2+}$-activated Cl$^-$ channel function, may not necessarily cause a loss of physiological functions. This has been demonstrated recently in Ano2 knockout animals, in which loss of Ca$^{2+}$-activated Cl$^-$ currents was shown to be dispensable for olfaction (Billig et al. 2011). Loss of the modulatory function of CaCC in olfactory receptors is probably compensated by the downstream neuronal circuit.

**Activation of Ano1 by Ca$^{2+}$**

Anoctamin 1 is activated by a rise in intracellular Ca$^{2+}$, and different splice variants of Ano1 seem to differ regarding their Ca$^{2+}$ sensitivity (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008; Ferrera et al. 2009). We found that the Ca$^{2+}$-binding protein, calmodulin, but not the calmodulin-dependent kinase, CAMKII, is essential for activation of Ano1 overexpressed in HEK 293 cells (Tian et al. 2011). Although phosphorylation by CAMKII is not required to open the channel, ATP needs to be present on the cytosolic side of the membrane (Tian et al. 2011). The calmodulin dependence and ATP dependence of Ano1 are reminiscent of Ca$^{2+}$-dependent KCNN4 K$^+$ channels, which also require both factors for complete activation (Pedersen et al. 1999). The Hartzell team demonstrated recently that Ano1 is gated both by Ca$^{2+}$ and by strong depolarization, even in the complete absence of Ca$^{2+}$, although gating by both Ca$^{2+}$ and voltage are very closely coupled. In this respect, Ano1 behaves similar to large-conductance Ca$^{2+}$-activated (BK) K$^+$ channels (Latorre & Brauchi, 2006).

**Compartmentalization of Ca$^{2+}$ signals is important for activation of Ano1**

When expressed in HEK 293 cells, Ano1 is well activated by stimulation of purinergic P2Y$_2$ receptors with ATP. Replacement of extracellular Cl$^-$ by gluconate shifts the reversal potential to depolarized membrane voltages (Fig. 2A). It was shown in the initial paper by Yang et al. (2008) that coexpression of P2Y$_2$ receptors together with Ano1 largely augments activated ion currents. We found that at a maximal agonist concentration of 100 $\mu$M ATP, increase of intracellular [Ca$^{2+}$], as measured by fura-2, was not further increased by additional expression of purinergic P2Y$_2$ receptors, although activation of whole-cell currents by ATP was greatly increased (Fig. 2B–D; Yang et al. 2008). There may be a Ca$^{2+}$-independent coupling between P2Y$_2$ receptors and Ano1 (Tian et al. 2011), although we did not find evidence for a direct physical interaction (Fig. 2E).

It could be the local Ca$^{2+}$ signalling in close proximity to Ano1 that matters for channel gating rather than the global cytosolic Ca$^{2+}$ (Fig. 2F, upper panel). Local endoplasmic reticulum (ER) Ca$^{2+}$ release and influx through store-operated Ca$^{2+}$ channels determine local intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Expression of anoctamins may intensify local Ca$^{2+}$ signals, perhaps by tethering components of intracellular Ca$^{2+}$ signalling directly to anoctamin (Kunzelmann et al. 2011a). These true local Ca$^{2+}$ signals may not be spatially resolved in fura-2 measurements (Barro-Soria et al. 2009). The idea of local Ca$^{2+}$ signals being relevant for activation of Ano1 was supported by experiments performed in *Xenopus laevis* oocytes, which express endogenous Ano1 (Schroeder et al. 2008). We expressed P2Y$_2$ receptors, and Ano1 currents

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**Figure 1. Anoctamin 1 (Ano1) knockout mice show multiple defects**

A. Ano1 knockout animal (−/−) and wild-type (+/+) littermate. B. animal studies were conducted according to the German laws on protection of animals. Mice were killed under CO$_2$ narcosis by cervical dislocation. Tracheas were dissected, opened longitudinally on the side opposite to the cartilage-free zone and were transferred immediately into an ice-cold buffer solution (mm: NaCl, 145; KH$_2$PO$_4$, 0.4; K$_2$HPO$_4$, 1.6; d-glucose, 6; MgCl$_2$, 1; and calcium gluconate 1.3; pH 7.4). Contraction of longitudinally opened tracheas in response to stimulation with carbachol (100 $\mu$m) was measured in a humidified and heated (37°C) chamber. Contraction was measured as the reduction of the diameter of the tracheas and was found to be reduced in Ano1−/− animals when compared with wild-type (+/+) animals. # Significant difference (P < 0.05, Student’s unpaired t test). Number of experiments is given in parentheses above each bar.
were activated by 100 μM ATP. We found that activation of Ano1 by ATP was hardly affected by incubation with the Ca²⁺ chelator BAPTA-AM, but was largely inhibited by removal of extracellular Ca²⁺ (Fig. 3A and B). Notably, Ca²⁺ store depletion with 10 μM cyclopiazonic acid or thapsigargin did not affect ATP-activated Cl⁻ currents (Fig. 3C–E). Hartzell (1996) identified Ca²⁺-activated Cl⁻ currents in Xenopus oocytes and described two different macroscopic currents in these cells, namely one activated selectively by Ca²⁺ influx and the other activated by both influx and release of Ca²⁺ from ER Ca²⁺ stores. According to the present results, we suggest that Ca²⁺ influx is highly relevant for receptor-mediated activation of Ano1.

It is well known that a mild increase in intracellular Ca²⁺ leads to outwardly rectifying Cl⁻ currents, while intracellular Ca²⁺ concentrations above 1 μM activate Cl⁻ currents with a rather linear current–voltage relationship (Hartzell, 1996; Kuruma & Hartzell, 2000).

The Hartzell team demonstrated a voltage dependence of Ca²⁺ activation for Ano1 channels (Xiao et al. 2011). Calcium ionophores, such as ionomycin (at 1 μM), are known to increase [Ca²⁺]i well beyond 1 μM (as measured by fura-2). These ionophores are regularly used to calibrate Ca²⁺ signals (Silver, 1998). In Xenopus oocytes overexpressing Ano1 and P2Y₂ receptors, however, whole-cell currents activated by 1 μM ionomycin were for the most part outwardly rectifying, while 100 μM ATP activated linear currents, suggesting higher local Ca²⁺ concentrations (Fig. 3F; Kunzelmann et al. 2011b). Activation of endogenous CaCC by stimulation of P2Y₂ receptors (no additional expression of Ano1) produced outwardly rectifying currents, which were completely inhibited by removal of extracellular Cl⁻ (Fig. 3G). In contrast P2Y₂ and Ano1 coexpressing oocytes demonstrated linear currents upon ATP stimulation, and removal of extracellular Cl⁻ abolished Cl⁻ (outward) currents, while inward currents remained (Fig. 3G).

![Figure 2](image-url)

**Figure 2. Purinergic P2Y₂ receptors and Ano1 form a functional unit**

Whole-cell currents (patch clamping) and intracellular Ca²⁺ concentrations (fura-2) were measured in HEK 293 cells expressing Ano1 and P2Y₂ receptors. *A*, current–voltage relationships obtained in whole-cell patch clamp experiments with Ano1 currents expressed in HEK 293 cells. Removal of extracellular Cl⁻ by gluconate (gluc) inhibits Ano1 currents. *B* and *C*, summary of individual experiments and bar graph of Ano1 conductances obtained from HEK 293 cells in the absence or presence of coexpressed P2Y₂ receptors. * Significant activation of whole-cell currents by ATP (P < 0.05, Student's paired t test). # Significant difference between absence and presence of P2Y₂ receptors (P < 0.05, Student's unpaired t test). *D*, ATP (100 μM) induced Ca²⁺ signals in the absence or presence of additional P2Y₂ receptors. *E*, Western blot of Ano1 [molecular weight 110 (unglycosylated) and 150 kDa (glycosylated)] expressed in HEK 293 cells. Cells expressed only P2Y₂ receptor (left lane) or P2Y₂ receptor and Ano1 (middle and right lane). Anoctamin 1 was detected only after immunoprecipitation with Ano1 antibody (middle lane), but was not co-immunoprecipitated with P2Y₂ receptor (P2Y₂ immunoprecipitation; right lane). *F*, model for localized Ca²⁺ signalling augmented by the presence of Ano1. Number of experiments is given in parentheses.
Figure 3. Additional Ca\(^{2+}\) influx in Ano1-expressing oocytes

Oocytes were harvested from *Xenopus laevis* according to German regulations governing animal experiments. Oocytes were defolliculated for 1 h at 18°C with collagenase in OR2 solution (in mmol l\(^{-1}\): NaCl, 82.5; KCl, 2; MgCl\(_2\), 1; and Hepes, 5; pH 7.55) and then injected with 10 ng (47 nl double-distilled water) of cRNA encoding P2Y\(_2\) receptor and Ano1. A, whole-cell Ano1-currents measured in double-electrode voltage-clamp experiments in *Xenopus laevis* oocytes. Removal of extracellular Ca\(^{2+}\) but not the Ca\(^{2+}\) chelator BAPTA (50 μM) abolished ATP activation (P2Y\(_2\)) of Ano1. B, summary of the conductances obtained from experiments described in A. C and D, Ca\(^{2+}\) release from the endoplasmic reticulum store by inhibition of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase Ca\(^{2+}\) pump with cyclopiazonic acid (CPA; 10 μM) did not interfere with activation of Ano1. E, effect of thapsigargin (Thapsi; 10 μM), CPA, inhibition of Erk1/2 by U0126 (20 μM; Almaca et al. 2009) and the sulfhydryl reagent MTSET (5 mM; Yang et al. 2008) on ATP-activated Ano1 currents. F, activation of Ano1 by ATP (100 μM) or ionomycin (IONO; 1 μM). G, Ano1 currents activated by ATP and removal of extracellular Cl\(^{-}\) (gluconate) in oocytes expressing P2Y\(_2\) receptors or overexpressing P2Y\(_2\) receptors and Ano1. An extracellular free Ca\(^{2+}\) concentration of 1 mM was maintained even in the presence of gluconate. * Significant difference (P < 0.05, Student’s paired t test). # Significant difference (P < 0.05, Student’s unpaired t test). Number of experiments is given in parentheses.
Why is this? Both endogenous and overexpressed Ano1 are activated through receptor-mediated Ca\(^{2+}\) increase and Erk1/2 phosphorylation (Tian et al. 2011), and should therefore behave in a similar manner. The difference could be explained by a higher local \([\text{Ca}^{2+}]_i\), in overexpressing cells, upon receptor stimulation (causing a linearization of the current–voltage relationship). Thus, additional (overexpressed) Ano1 channels would increase intracellular Ca\(^{2+}\) signalling. Alternatively, Ano1 itself causes a cation conductance (Fig. 2F; Kunzelmann et al. 2011b).

**Epithelial CaCC: is it all Ano1?**

The Verkman team recently claimed that Ano1 is only a minor component of CaCC in airway and intestinal epithelial cells (Namkung et al. 2011). This conclusion was based on results from a novel class of small-molecule inhibitors that fully blocked Ano1 chloride currents, but did not interfere with calcium signalling. The lack of specific inhibitors for CaCC and Ca\(^{2+}\)-independent Cl\(^{-}\) channels, such as CFTR, has always been a problem. Typically, inhibitors such as DIDS, 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid, niflumic acid and tamoxifen have been used in higher micromolar ranges as inhibitors for CaCC and CFTR (Hartzell et al. 2005). The Verkman team, meanwhile, developed more specific and potent inhibitors for CFTR and CaCC (Ma et al. 2002; de la Fuente et al. 2007). Interestingly, Ano1 currents overexpressed in HEK 293 cells are remarkably sensitive to ‘classical’ CaCC inhibitors, i.e. in general, higher concentrations are required to inhibit endogenous CaCC in epithelial cells (Hartzell et al. 2005; Fig. 4A). In our hands, the most potent inhibitor of endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channels is 6-t-butyl-2-(furan-2-carboxamido)-4,5,6,7-tetrahydrobenzo[b] thiophene-3-carboxylic acid (AO1), which was developed in the Verkman laboratory (de la Fuente et al. 2007). We found that AO1 also potently inhibited Ano1 expressed in HEK 293 cells, although AO1 also has some additional unwanted effects on intracellular Ca\(^{2+}\); 10 \(\mu\)M AO1 increased intracellular Ca\(^{2+}\) levels, while in the presence of AO1 the peak and plateau increase of \([\text{Ca}^{2+}]_i\) by ATP were reduced (Fig. 4A and B).

![Figure 4. Effects of CaCC inhibitors on Ano1 and intracellular Ca\(^{2+}\)](image)

Whole-cell currents (patch clamping) and intracellular Ca\(^{2+}\) concentrations (fura-2) were measured in HEK 293 cells expressing Ano1. A, inhibition of whole-cell Ano1 currents in HEK 293 cells by different blockers (all 10 \(\mu\)M A01, 6-t-butyl-2-(furan-2-carboxamido)-4,5,6,7-tetrahydrobenzo[b] thiophene-3-carboxylic acid; TA, tannic acid, NFA, niflumic acid; DIDS, 4, 4′-disothiocyanostilbene-2,2′-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid; and Tamox, tamoxifen). B and C, ATP-induced increase in \([\text{Ca}^{2+}]_i\) and effect of AO1 (20 \(\mu\)M) on \([\text{Ca}^{2+}]_i\). # Significant difference (\(P < 0.05\), Student’s unpaired t test). Number of experiments is given in parentheses.

![Figure 5. Lack of expression of Ano1 in human nasal and colonic epithelium](image)

A and B, RT-PCR analysis of anoctamin expression in cultured airway epithelial cells (16HBE) and freshly isolated human nasal epithelium and rectal biopsies. Anoctamin 1 is clearly expressed in cultured human airway cells, but is missing in freshly isolated tissues, which instead express Ano6 and Ano9, apart from faint expression of Ano5 and Ano8. All bands were of correct size, and sequences were confirmed by DNA sequencing. pAno1, plasmid containing Ano1 sequence.
Thus, it is currently unknown whether endogenous epithelial CaCC is mainly due to Ano1 or whether other anoctamins or unrelated proteins contribute to endogenous CaCC. In contrast to Namkung et al. (2011), we found significant inhibition of CaCC by small interfering RNA knockdown of Ano1 in human colonic cancer (HT29), pancreatic epithelial (CFPAC) and squamous cell carcinoma (Cal-33) cells (data not shown). Also, the Galietta team found Ano1 associated with native calcium-activated chloride channels (Ferrera et al. 2009). Finally, in Ano1 null mice Ca\(^{2+}\)-activated Cl\(^{-}\) currents are absent in salivary glands, trachea, exocrine pancreas and large intestine (Ousingsawat et al. 2009). Taken together, good evidence is provided that Ano1 is responsible for endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) channels, although other proteins, including members of the anoctamin family, may have a role in shaping tissue-specific properties of CaCC.

**Anoctamin 6 produces Cl\(^{-}\) currents**

Although Ano1 was shown to be in charge of Ca\(^{2+}\)-activated Cl\(^{-}\) currents in basically every tissue examined, we could not detect Ano1 expression in a recent RT-PCR analysis of freshly isolated human nasal and colonic epithelium. In contrast, Ano1 was well detected in cultured 16HBE airway epithelial cells (Fig. 5A). According to

![Figure 6. Anoctamin 6 currents and regulatory volume decrease in HeLa cells](image)

**A**, whole-cell patch clamp experiments performed in HeLa cells. Current–voltage relationships obtained in experiments with control HeLa cells (pcDNA3), Ano6- and Ano6-D408G-overexpressing cells. Enhanced baseline whole-cell currents are inhibited by AO1 (20 \(\mu\)M). **B**, RT-PCR analysis of anoctamin expression in HeLa cells. **C** and **D**, calcein fluorescence detected in HeLa cells as a measure for cell volume. Hypotonic cell swelling and recovery from swelling (regulatory volume decrease) were measured by calcein fluorescence in control and Ano6-expressing HeLa cells. * Significant difference (\(P < 0.05\), Student's paired t test). Number of experiments is given in parentheses.
earlier studies on native (non-cultured) human nasal epithelium, a small but significant \( \text{Ca}^{2+} \) (ATP)-activated \( \text{Cl}^- \) secretion was detected, and therefore expression of Ano1 would be expected, unless another anoctamin is in charge of \( \text{Ca}^{2+} \)-dependent \( \text{Cl}^- \) secretion (Mall \textit{et al.} 2000; Rowe \textit{et al.} 2011). Also, it cannot be entirely excluded that ATP stimulation activates primarily basolateral \( \text{Ca}^{2+} \)-dependent \( \text{K}^+ \) channels, thereby increasing the driving force for \( \text{Cl}^- \) exit through apical CFTR and Ano1-independent \( \text{Cl}^- \) channels.

A more detailed analysis of Ano1 expression in human native large intestine and nasal epithelium indicated a complete lack of Ano1, but demonstrated predominant expression of Ano6, apart from faint expression of Ano5 and Ano8, which nevertheless may contribute to CaCC (Fig. 5B). These results put some doubt into the role of Ano1 in human nasal epithelium and large intestine; therefore, a more detailed analysis of the different parts of the human respiratory tract will be required before any final conclusion can be drawn. The results, however, also challenge the question whether Ano6 generates a \( \text{Ca}^{2+} \)-dependent \( \text{Cl}^- \) current. This question became even more challenging after a recent report demonstrating that Ano6 is a scramblase (Suzuki \textit{et al.} 2010). In an earlier study, we found a small but detectable \( \text{Ca}^{2+} \)-activated \( \text{Cl}^- \) current in Ano6-transfected Fisher rat thyroid (FRT) cells (Schreiber \textit{et al.} 2010). Here, we overexpressed in HeLa cells both Ano6 and D408G-Ano6, which had been...
reported as a Ca$^{2+}$-hypersensitive mutant regarding the scramblase activity of Ano6 (Suzuki et al. 2010). We found that overexpression of Ano6 results in an enhanced baseline current that is inhibited by AO1 (Fig. 6A). Also, D408G-Ano6-expressing cells demonstrated enhanced AO1-sensitive whole-cell currents, although these currents only developed within a few minutes after establishing the whole-cell configuration. Control HeLa cells transfected with pcDNA3 did not show AO1-inhibited whole-cell currents, although these cells express endogenous Ano6, as demonstrated by RT-PCR (Fig. 6B). Regulatory volume decrease after hypotonic cell swelling was not changed by overexpression of Ano6 (Fig. 6C and D). In contrast to HeLa cells, HEK 293 cells demonstrated enhanced regulatory volume decrease upon overexpression of Ano6 or Ano6-D408G (Fig. 7) and showed reduced regulatory volume decrease after small interfering RNA knockdown of endogenous Ano6 (Almaça et al. 2009). Thus the contribution of Ano6 to Cl$^{-}$ conductance and regulatory volume decrease might be cell specific.

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