Anoctamins are a family of Ca\(^{2+}\)-activated Cl\(^{-}\) channels

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Summary
Anoctamin 1 (Ano1; TMEM16A) and anocatin 2 (Ano2; TMEM16B) are novel Cl\(^{-}\) channels transiently activated by an increase in intracellular Ca\(^{2+}\). These channels are essential for epithelial Cl\(^{-}\) secretion, smooth muscle peristalsis and olfactory signal transduction. They are central to inherited diseases and cancer and can act as heat sensors. Surprisingly, another member of this protein family, Ano6, operates as a Ca\(^{2+}\)-activated phospholipid scramblase, and others were reported as intracellular proteins. It is therefore unclear whether anoctamins constitute a family of Ca\(^{2+}\)-activated Cl\(^{-}\) channels, or are proteins with heterogeneous functions. Using whole-cell patch clamping we demonstrate that Ano4–10 are all able to produce transient Ca\(^{2+}\)-activated Cl\(^{-}\) currents when expressed in HEK293 cells. Although some anoctamins (Ano1, 2, 4, 6, 7) were found to be well expressed in the plasma membrane, others (Ano8, 9, 10) show rather poor membrane expression and were mostly retained in the cytosol. The transient nature of the Cl\(^{-}\) currents was demonstrated to be independent of intracellular Ca\(^{2+}\) levels. We show that inactivation of Ano1 currents occurs in the continuous presence of elevated Ca\(^{2+}\) concentrations, possibly by calmodulin-dependent kinase. The present results demonstrate that anoctamins are a family of Ca\(^{2+}\)-activated Cl\(^{-}\) channels, which also induce permeability for cations. They may operate as Cl\(^{-}\) channels located in the plasma membrane or in intracellular compartments. These results increase our understanding of the physiological significance of anoctamins and their role in disease.

Key words: CaCC, TMEM16A, TMEM16B, TMEM16D, TMEM16E, TMEM16F, TMEM16G, TMEM16H, TMEM16J, TMEM16K, Ca\(^{2+}\)-activated Cl\(^{-}\) channels, CaCC, Calmodulin, Anoctamin

Introduction
Ca\(^{2+}\)-activated Cl\(^{-}\) currents (CaCC) are abundant and are present in nearly every cell type, where they fulfill very different functions (Hartzell et al., 2005). It is now well accepted that TMEM16A (anoctamin 1, Ano1) forms the plasma membrane localized Ca\(^{2+}\)-activated Cl\(^{-}\) channel (Yang et al., 2008; Schroeder et al., 2008; Caputo et al., 2008). Voltage- and calcium-dependent gating of Ano1 has been examined in detail, and is linked to the first intracellular loop (Xiao et al., 2011). Ano1 is essential for Cl\(^{-}\) secretion in a number of epithelial tissues (Ousingsawat et al., 2009; Rock et al., 2009), for smooth muscle contraction (Thomas-Gatewood et al., 2011; Manoury et al., 2010), the function of nociceptive neurons and smooth muscle pacemaker cells (Liu et al., 2010; Hwang et al., 2009; Huang et al., 2009). Ano2, the closest relative of Ano1, has been shown to form a CaCC in olfactory receptors (Sagheddu et al., 2010; Billig et al., 2011).

The family of anoctamins consists of 10 different proteins, but only Ano1, Ano2 and Ano6 have been examined in more detail (Ferrera et al., 2010; Billig et al., 2011; Kunzelmann et al., 2009). For Ano6 a dual role as Fas ligand-activated Cl\(^{-}\) channel and phospholipid scramblase has been described (Martins et al., 2011; Suzuki et al., 2010). However, whether Ano3–10 also produce Ca\(^{2+}\)-activated Cl\(^{-}\) currents similar to Ano1 and Ano2, is currently unclear. A recent report claimed that Ano3–7 are intracellular proteins (Duran et al., 2012), although we demonstrated earlier that most anoctamins overexpressed in HEK293 cells can be detected in the cell membrane (Schreiber et al., 2010).

Anoctamins do not show any obvious homology to other ion channels. Ano1 contains eight predicted transmembrane helices, intracellular NH\(_2\)- and COOH-ends and a pore, formed by the fifth and sixth transmembrane helices, containing a p-loop dipping back into the membrane (Yang et al., 2008). The other anoctamins (2–10) show a high degree of structural similarity, which is particularly obvious for the putative pore region (Kunzelmann et al., 2009). Apart from some indirect evidence showing enhanced ATP-induced halide permeability in Ano6- and Ano7-expressing cells (Schreiber et al., 2010), it is not clear whether other anoctamins also produce Ca\(^{2+}\)-activated Cl\(^{-}\) currents. We demonstrated earlier that Ano6 produces an outwardly rectifying chloride channel, which is activated during stimulation of Fas receptors upon induction of apoptosis (Martins et al., 2011). Notably, at high intracellular Ca\(^{2+}\) concentrations as induced by Ca\(^{2+}\) ionophores, Ano6 was reported to operate as a membrane phospholipid scramblase (Suzuki et al., 2010). In contrast to Ano1 and Ano2, Ano6 Cl\(^{-}\) currents were not activated by physiological Ca\(^{2+}\) concentrations (Martins et al., 2011). As demonstrated in the present report, high intracellular Ca\(^{2+}\) levels are required to activate Ano6. Although plasma membrane expression of some anoctamins is very poor, the present experiments also demonstrate for the first time that essentially all anoctamins are able to produce Cl\(^{-}\) currents when activated by an increase in [Ca\(^{2+}\)]. We speculate that anoctamins may operate as plasma membrane or intracellular Cl\(^{-}\) channels.

Results and Discussion
Activation of Ano1, 4, 5, 6, 7, 8, 9 and 10 by ATP-induced increase in intracellular Ca\(^{2+}\)
Previous studies have demonstrated that Ano1 can be activated by stimulation of G-protein-coupled receptors, such as purinergic
P2Y<sub>2</sub> receptors, which increase [Ca<sup>2+</sup>]<sub>i</sub> (Yang et al., 2008). Because Ano2 has also been demonstrated to operate as a Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel (Sagheddu et al., 2010; Billig et al., 2011), we asked whether other anoctamins are also able to produce Ca<sup>2+</sup>-activated Cl<sup>-</sup> currents. To that end we coexpressed different anoctamins together with P2Y<sub>2</sub> receptors in HEK293 cells, which were stimulated by 10 μM ATP, in the presence of a cytosol-like pipette filling solution and extracellular Ringer solution (see the Materials and Methods). We observed that overexpressed Ano1 and Ano6 produce significant whole-cell Cl<sup>-</sup> currents, even in the absence of ATP stimulation (Kunzelmann et al., 2011b). In cells expressing Ano1, 4, 7, 8, 9 and 10, whole-cell currents of variable magnitudes were activated by ATP (Fig. 1). Current activation by ATP was fast (below 1 s) for Ano1, but was delayed for the other anoctamins. It is currently unclear whether this is due to a reduced sensitivity towards intracellular Ca<sup>2+</sup>, since intracellular Ca<sup>2+</sup> levels are increased equally fast in all cells by either ATP or ionomycin, when assessed by Fura-2 (Fig. 2). Increase of [Ca<sup>2+</sup>]<sub>i</sub> may not only activate the Cl<sup>-</sup> channel, but may also trigger inhibition of anoctamins via CAMKII, which may actually counteract further activation. CAMKII inhibition was suggested in a recent study (Tian et al., 2011) and is also demonstrated in the present paper for both, Ano1 and Ano6. For all anoctamins except of Ano4, membrane voltages were depolarized through stimulation of purinergic receptors and increase of [Ca<sup>2+</sup>]<sub>i</sub>, which is expected upon increase of a Cl<sup>-</sup> conductance along with cation influx through store operated Ca<sup>2+</sup> channels (Fig. 1B). Surprisingly, expression of Ano6 hyperpolarized the membrane voltage of non-stimulated cells, which is probably due to its partial permeability for K<sup>+</sup> ions as described further below. We wondered whether largely enhanced baseline Cl<sup>-</sup> currents in Ano1-expressing cells are due to activation of by the baseline Ca<sup>2+</sup> concentration of 100 nM (Fig. 2A). In fact, 30 min pre-incubation with the Ca<sup>2+</sup>-chelator BAPTA-AM (25 μM) abolished this baseline Cl<sup>-</sup> conductance (Fig. 2C). We hypothesize that an inhibitory factor or additional protein is missing that keeps overexpressed channels closed under control conditions. As described below, this unknown protein could be another anoctamin, like Ano9, which may be able to heterooligomerize with Ano1 (Fig. 6G). Moreover, since expression of anoctamins also induced a cation permeability (cf. below), we exclude a possible contribution of endogenous Ca<sup>2+</sup>-activated SK4 K<sup>+</sup> channels: Activation of whole-cell currents in Ano1-expressing HEK293 cells by ionomycin was identical in the absence or presence of 10 μM clotrimazole, an inhibitor of Ca<sup>2+</sup>-activated K<sup>+</sup> channels (the number of experiments is given in brackets). Different from control (P<0.05), ANOVA. *Significant activation (P<0.05), paired t-test.

**Fig. 1. Activation of anoctamins by ATP.** (A) Continuous recordings of whole-cell currents measured in HEK293 cells expressing different human anoctamins and activation by 10 μM ATP. At intervals cells were voltage clamped from −50 mV to +50 mV in steps of 10 mV. (B) Summary of the calculated peak conductances and membrane voltages of transiently activated anoctamins. Values are means ± s.e.m.; the number of cells used is given in brackets. Different from mock (P<0.05), ANOVA. *Significant activation (P<0.05), paired t-test.

**Fig. 2. Ca<sup>2+</sup> dependence of anoctamins.** (A, B) Time course for (A) ATP-induced (10 μM; summary of 30 experiments) and (B) ionomycin-induced (1 μM; summary of 16 experiments) increase in [Ca<sup>2+</sup>]<sub>i</sub>, in HEK293 cells, as detected by ratiometric Fura-2 fluorescence. (C) Summary of baseline conductance and activated whole-cell conductances obtained in Ano1-expressing HEK293 cells under control conditions and after pre-incubation with 25 μM BAPTA-AM. (D) Summary of ionomycin-activated whole-cell conductance in the presence or absence of 10 μM clotrimazole, an inhibitor of Ca<sup>2+</sup>-activated K<sup>+</sup> channels (the number of experiments is given in brackets).

**Activation of anoctamins by a Ca<sup>2+</sup> ionophore**

Stimulation by 10 μM ATP raised intracellular Ca<sup>2+</sup> levels to about 1 μM, which is mainly due to release of Ca<sup>2+</sup> from intracellular ER stores (Fig. 2A) (Barro-Soria et al., 2010). This Ca<sup>2+</sup> increase may not be sufficient to activate every member of the anoctamin family. We therefore stimulated the cells with the...
Ca²⁺ ionophore ionomycin (1 μM), which raised [Ca²⁺], in a more sustained manner to levels well above 1 μM (Fig. 2B). In fact whole-cell currents could be activated in cells expressing Ano5 or Ano6, when stimulated by ionomycin, suggesting that these channels require higher and maybe more sustained Ca²⁺ levels to be activated (Fig. 3A). Close to the plasma membrane ionomycin raises [Ca²⁺], probably to much higher levels than suggested by Fura-2 measurements. We examined the Ca²⁺ dependence of Ano6 by varying cytosolic (patch pipette) Ca²⁺ concentrations and detected substantial activation of Cl⁻ currents only at concentrations as high as 10 μM or larger (Fig. 3D). Moreover, Ano6 shows little inactivation during continuous stimulation with 1 μM ionomycin (Fig. 3B). Anion selectivity was similar for Ano1, 4, 6 and 10 and was I⁻>Br⁻>Cl⁻>HCO₃⁻, suggesting a low field strength anion selectivity. HCO₃⁻ was conducted surprisingly well by Ano6 (Fig. 3C). It is therefore entirely possible that Ano6 serves as a channel for bicarbonate secretion in airways and other epithelial tissues. Notably, at very high [Ca²⁺], of 0.1 and 1 mM, whole-cell Cl⁻ currents were also activated in mock-transfected cells, which express Ano6 endogenously (Fig. 3D,E). Replacement of extracellular Cl⁻ with gluconate strongly inhibited whole-cell currents and depolarized membrane voltages, indicating activation of whole-cell Cl⁻ currents at high [Ca²⁺], in both mock-transfected and Ano6-overexpressing cells (Fig. 3D–F). We speculate that endogenous anoctamins may be activated only at very high [Ca²⁺]. (Almaça et al., 2009). Indeed when endogenous Ano6 (together with Ano1, 8 and 9) were knocked down simultaneously by siRNA, we found reduced baseline currents. Currents activated by 1 mM [Ca²⁺] in the patch pipette filling solution were largely attenuated (Fig. 3G). Because of the lack of suitable antibodies we assessed knockdown of anoctamins by real time RT-PCR. Expression levels (relative to β-actin) were very different for the endogenous anoctamins, with anoctamin 6 being the most abundant anoctamin (n=3 for all): Ano1 1.4×10⁻⁶ (downregulation by 52%); Ano6 3.1×10⁻⁴ (downregulation by 82%); Ano8 1.3×10⁻⁴ (downregulation by 42%); Ano9 2.0×10⁻⁸ (downregulation by 76%). Thus Ano6 and maybe Ano8 have probably the largest impact on endogenous Ca²⁺-activated Cl⁻ conductance. It remains currently unclear why endogenous anoctamins require such high [Ca²⁺] to be activated.

In more than 20 cell lines and in oocytes we examined endogenous expression of anoctamins. Ano6 and Ano8 were always expressed most abundantly (Kunzelmann et al., 2009). Thus, there is no ‘empty’ system without background expression of endogenous anoctamins. However, given the low levels for endogenous anoctamins compared to overexpressed proteins (about 1000 times higher according to real time RT-PCR analysis), possible heterooligomerization of endogenous and overexpressed proteins does not appear to be a major problem in the present study.

**Fig. 3. Activation of anoctamins by ionomycin.** (A) Summary of the calculated ionomycin (1 μM)-activated peak conductances and measured (currents clamp) membrane voltages of HEK293 cells expressing anoctamins. (B) Whole-cell currents activated by ionomycin in Ano6-overexpressing cells. (C) Mean current/voltage relationships for Ano6 whole-cell currents, with different anions (Cl⁻, I⁻, Br⁻, HCO₃⁻; Bic) present in the bath solution. (D) Concentration-response curve for the Ca²⁺ activation of whole-cell conductance in Ano6-overexpressing and mock-transfected HEK293 cells. (E) Continuous original recordings of the whole-cell conductance (left, black: mock; right, red: Ano6) activated by 1 mM Ca²⁺ in the patch pipette solution, and effects of removal of extracellular Cl⁻ (5Cl⁻). At intervals, cells were voltage clamped from −50 mV to +50 mV in steps of 10 mV. (F) Summaries of the calculated whole-cell conductances and measured membrane voltages in the presence of 0.1 and 1 mM Ca²⁺, before and after removal of extracellular Cl⁻ (5Cl⁻). (G) Original current recording and summary of the effects of 1 mM Ca²⁺ on HEK293 cells treated with siRNA for Ano1, 6, 8 and 9. Values are means ± s.e.m.; the number of cells used is given in brackets.

**Contribution of nonselective currents and gating by cations**

Cl⁻ currents activated by ionomycin or high intracellular (pipette) Ca²⁺ concentrations demonstrated rather linear current/voltage relationships (Fig. 4). We compared currents activated by ionomycin in the presence of extra-and intracellular cations (cytosol-like pipette filling solution and bath Ringer solution), with currents detected in the absence of extra- and intracellular cations (patch pipette 132 mM CsCl, bath 132 mM NMDGCl; see the Materials and Methods). In general, replacing intracellular K⁺ by Cs⁺ and extracellular Na⁺ by impermeable NMDG⁺ markedly reduced whole-cell currents produced by some anoctamins (Fig. 4). We further examined possible cation permeability of several anoctamins (1, 6 and 10) by replacing intracellular K⁺ and extracellular Na⁺ by NMDG⁺ separately (Fig. 5). Indeed we found that removal of intracellular K⁺ depolarized membrane voltage and reduced outward currents, while removal of bath Na⁺ hyperpolarized membrane voltage and reduced inward (and outward) currents (Fig. 5). Particularly in Ano6-expressing cells a permeability for K⁺ ions was detected. Indirect evidence for cation conductance of anoctamins has already been provided earlier (Kunzelmann et al., 2011b; Kunzelmann et al., 2012). Moreover, anoctamins are also permeable for Ca²⁺, as reported for Ano6 (Yang et al., 2011). Taken together these results suggest that anoctamins are either
poorly selective for anions or lead to parallel activation of a cation conductance.

**Inactivation of anoctamins**

Increase in intracellular Ca\(^{2+}\) activates anoctamins only transiently. On the other hand, overexpression of Ano1 in HEK293 and other cell types leads to channels that are partially active even under baseline Ca\(^{2+}\) concentrations of 100 nM (Figs 1, 2) (Kunzelmann et al., 2011b). We observed that during transient activation/deactivation of Ano1 whole-cell Cl\(^{-}\) currents declined even below the initial high current levels, This was observed during continuous stimulation by ATP (10 \(\mu\)M) or ionomycin (1 \(\mu\)M), or when 1 \(\mu\)M Ca\(^{2+}\) was provided through the patch pipette filling solution (1 \(\mu\)M; Fig. 6A). This suggests that increase in [Ca\(^{2+}\)], initially activates the channel but then triggers inactivation by a Ca\(^{2+}\)-dependent process. Spontaneous current inactivation was not due to a loss of ATP or calmodulin, since we also observed inactivation when both components (3 mM ATP, 2 \(\mu\)M calmodulin) were present in the patch pipette. Because Ca\(^{2+}\)/calmodulin-dependent kinase II (CAMKII) inhibits overexpressed Ano1 currents (Tian et al., 2011), we hypothesized that an increase of Ca\(^{2+}\) may activate CAMKII, which in turn inhibits Ano1. In fact, in the presence of the CAMKII blocker KN62 (5 \(\mu\)M), inactivation of the channel was significantly attenuated (Fig. 6B). Similar to Ano1, also Ano6 contains four putative CAMKII phosphorylation sites in the N-terminus (supplementary material Fig. S2). Thus inhibition of CAMKII by 10 \(\mu\)M KN62 also increased activation of Ano6 by 1 \(\mu\)M ionomycin [$\Delta\text{G}_{\text{ano6}} = 29.6 \pm 3.6$ (w/o KN62) versus $43.3 \pm 4.5$ nS (w/KN62); \(n=5\)].

Moreover, when patch pipettes were loaded with pre-activated CAMKII (5000 U/ml), activation of whole-cell currents in Ano1-overexpressing cells was significantly reduced (Fig. 6C). Thus CAMKII inhibits overexpressed Ano1, which is reminiscent to inhibition of CaCC by CAMKII in smooth muscle cells (Greenwood et al., 2001). Along this line, a splice variant of Ano1 (ac-Ano1) had been reported to have a higher Ca\(^{2+}\) sensitivity than the splice form abc-Ano1 (Ferrera et al., 2009), which we used in our experiments. As reported by Ferrera et al., we also found that the channel is activated at [Ca\(^{2+}\)] below 100 nM (Fig. 6D). However, we also found that ac-Ano1 currents inactivated at lower Ca\(^{2+}\) concentrations than the splice form abc-Ano1 (Fig. 6D). We tried to further localize the molecular site within Ano1 that is in charge for its only transient activation. Since activation of Ano6 produces more sustained currents (Martins et al., 2011), we generated a chimeric protein in which the N-terminus of Ano1 was replaced with that of Ano6 (Ano1N6). Notably, whole-cell currents generated by the chimeric Ano1N6 channel were no longer transient, but generated a rather sustained current when activated by 1 \(\mu\)M ionomycin (Fig. 6E,F). This suggests that the N-terminus controls both activation and inactivation of anoctamins. Notably, potential binding sites for calmodulin have been found in the N-terminus of Ano1, along with a number of putative phosphorylation sites, including those for CAMKII (Kunzelmann et al., 2009).

**Overexpressed anoctamins behave differently**

The data presented above indicate that Ano6, 8 and 9, when overexpressed in HEK293 cells, produce Cl\(^{-}\) currents upon increase in intracellular Ca\(^{2+}\) by ionomycin. However, although all three paralogs are expressed endogenously in HEK293 cells,
no currents are activated in mock-transfected cells (Fig. 3A) (Almaça et al., 2009). However, when increasing [Ca\(^{2+}\)], to very high levels (≥10 μM), endogenous anoctamins were activated (Fig. 3B,E). This may be due to a lack of sufficient membrane expression of endogenous anoctamins. Endogenous anoctamins are expressed at much lower levels than overexpressed proteins, such as CAMKII. Alternatively, specific Ca\(^{2+}\) release and/or influx pathways need to be triggered to activate these endogenous anoctamins. The overall linear structures appear similar in all anoctamins (supplementary material Fig. S1) and an analysis of putative functional domains revealed a number of similar motifs and putative phosphorylation sites in all ten anoctamins (supplementary material Fig. S2). Notably, Ano1 exists as a stable dimer (Fallah et al., 2011; Sheridan et al., 2011), and may even form heterooligomeric proteins with other anoctamins (Schreiber et al., 2010). In the present study we therefore coexpressed different anoctamins and found that only coexpression of Ano9 inhibited Ano1 currents activated by ATP, suggesting a specific interaction of different anoctamin paralogs (Fig. 6G).

The present results suggest that endogenous and overexpressed anoctamins behave differently. We found earlier that calmodulin and 1-EBIO, a calmodulin-dependent activator of K\(^{+}\) channels, activate overexpressed Ano1 (Tian et al., 2011). In the present study we made use of a well-established technique to assess endogenous Ca\(^{2+}\)-activated Cl\(^{-}\) currents in HT29 cells stably expressing I\(^{-}\)-sensitive yellow fluorescent protein (YFP). Cellular YFP fluorescence is quenched by iodide influx from the bath solution (Verkman and Galietta, 2009) (Fig. 7A–C). Stimulation of purinergic receptors by ATP, dose dependently

Fig. 6. Increase in [Ca\(^{2+}\)], activates and subsequently inhibits anoctamins. (A) Activation and time-dependent decay of Ano1 whole-cell currents in HEK293 cells, when activated by ATP (10 μM), ionomycin (1 μM), and 1 μM Ca\(^{2+}\) in the patch pipette solution. (B) Time-dependent decay of whole-cell currents activated by 1 μM Ca\(^{2+}\) (patch pipette) in the absence or after pre-incubation with the inhibitor of CAMKII, KN62 (25 μM/2 hrs). (C) Activation of Ano1-whole-cell currents by ionomycin and effect of active CAMKII (5000 U/ml; 40 min) on current activation. (D) Ca\(^{2+}\)-dependent activation of the splice forms ac-Ano1 and abc-Ano1. (E) Sustained whole-cell conductances activated by 1 μM ionomycin in HEK293 cells expressing the chimeric protein Ano1N6 in which the N-terminus of Ano1 was replaced with that of Ano6. (F) Summary of the effect of ionomycin (1 μM) on whole-cell conductance and membrane voltage in Ano1N6-expressing HEK293 cells. (G) Summary of whole-cell conductances measured in HEK293 cells coexpressing different anoctamins. Values are means ± s.e.m.; the number of cells used is given in brackets. *Different from con or Ano1 (P<0.05); ANOVA. *Significant activation (P<0.05), paired t-test.

Fig. 7. Ca\(^{2+}\)-activated halide permeability in HT29 cells expressing endogenous Ano1. (A) Application of different concentrations of ATP leads to influx of extracellular I\(^{-}\) (20 mM) and concentration-dependent quenching of fluorescence in HT29 cells, stably expressing YFP. (B) Concentration–response curve for ATP-induced YFP-fluorescence quenching, indicating concentration-dependent activation of a Ca\(^{2+}\)-activated Cl\(^{-}\) conductance by ATP. (C) Summary of the inhibition of ATP-induced fluorescence quenching in HT29 cells by typical inhibitors of Ano1 (AO1 20 μM; NFA 10 μM; tannic acid TA 20 μM; DIDS 100 μM). (D) Incubation with 1-EBIO enhanced ATP-induced I\(^{-}\) uptake and quenching of YFP fluorescence in a dose-dependent manner. The Ca\(^{2+}\)-activated Cl\(^{-}\) conductance was inhibited at the end of the experiment by AO1 (20 μM). Cells were measured in the presence of clotrimazole (10 μM) to inhibit a possible contribution by the Ca\(^{2+}\)-dependent K\(^{+}\) channel SK4. (E) Inhibition of protein kinase C by pre-incubation with bisindolylmaleimide I (BIM; 0.1 μM/2 hrs) did not affect activation and the time course for inactivation of Ano1 overexpressed in HEK293 cells. (F) Inhibition of protein kinase C by BIM augmented endogenous Ano1 currents in HT29 cells. Values are means ± s.e.m.; the number of cells used is given in brackets. *Different from control (P<0.05), paired t-test.
influx and YFP quenching, indicating activation of a Ca\(^{2+}\)-dependent Cl\(^{-}\) conductance (Fig. 7A,B). ATP-activated Cl\(^{-}\) conductance was blocked by typical Ano1-inhibitors (Fig. 7C). Moreover, siRNA knockdown of Ano1 inhibited ATP-induced whole-cell currents in HT\(_{29}\) cells in another study (Tian et al., 2012). We examined the effects of 1-EBIO on Ca\(^{2+}\)-dependent Cl\(^{-}\) conductance in HT\(_{29}\) cells. However, even at a concentration of 1 mM 1-EBIO was unable to directly activate a Cl\(^{-}\) conductance (data not shown). It only slightly and dose dependently augmented ATP (1 \(\mu\)M)-activated Cl\(^{-}\) conductance (Fig. 7D). A number of 1-EBIO-related compounds (DCEBIO, riluzole, CBIQ, NS8593, CYPAA, NS309, NS4591) were examined in a dose-dependent manner. However, for none of them we found activation of a Cl\(^{-}\) conductance and receptor-mediated activation by ATP was not augmented (data not shown). We further performed experiments in a human airway epithelial cell line expressing the most common mutant form of CFTR, F508del-CFTR (CFBE/F508del-CFTR). We found: (1) that ATP (10 \(\mu\)M) activated a whole-cell Cl\(^{-}\) conductance of 3.5±0.4 nS (\(n=5\)), while direct application of 1-EBIO (100 \(\mu\)M) did not. (2) Activation of the whole-cell Cl\(^{-}\) conductance through ATP stimulation was slightly augmented by 0.7±0.08 nS (\(n=5\)) in the presence of 1-EBIO. Taken together these results demonstrate (i) that endogenous Ano1 behaves differently than overexpressed Ano1, and (ii) that 1-EBIO is probably not useful to directly activate Ca\(^{2+}\)-activated Cl\(^{-}\) secretion in airways of CF patients.

Finally, we detected differential regulation by protein kinase C of Ano1 overexpressed in HEK293 cells and endogenous Ano1 in HT\(_{29}\) cells. While inhibition of protein kinase C by preincubation with bisindolylmaleimide 1 (BIM; 0.1 \(\mu\)M/2 hrs) did not affect activation (by 1 \(\mu\)M ionomycin) of overexpressed Ano1 (Fig. 7E) (Tian et al., 2011), it augmented endogenous Ano1 currents in HT\(_{29}\) cells (Fig. 7F). Thus, properties and regulation of overexpressed anoctamins may not fully reflect those of native channels.

Most anoctamins are plasma membrane localized

A recent report did not find membrane expression or Ca\(^{2+}\)-activated Cl\(^{-}\) currents for Ano3–7 (Duran et al., 2012). This is in contrast to our earlier results which demonstrate plasma membrane expression for Ano1, 2, 5, 6, 7 and 9 when overexpressed in HEK293 and FRT cells (Schreiber et al., 2010). Moreover, it was shown recently that Ano6 is an essential component of outwardly rectifying Cl\(^{-}\) channels (ORCC) and, at the same time, operates as a membrane localized phospholipid scramblase (Martins et al., 2011; Suzuki et al., 2010). Moreover, also Ano5 and Ano7 were detected in the plasma membrane in earlier studies (Mizuta et al., 2007; Das et al., 2007). We re-examined plasma membrane expression of overexpressed anoctamins in HEK293 cells and, again, found clear membrane expression for Ano1, 4, 5, 6 and 7 and a weak membrane staining for Ano8, 9 and 10 (Fig. 8). We produced additional YFP fusion proteins for Ano6, 8 and 9. Life cell imaging of YFP fluorescence suggested (partial) membrane expression for Ano6, 8 and 9 (supplementary material Fig. S3). For Ano10 we managed to introduce a FLAG tag into the third extracellular domain and labeled non-permeabilized cells. The results show a spotted membrane expression of Ano10 (supplementary material Fig. S3). Thus the present data fully confirm those obtained in earlier studies (Schreiber et al., 2010).

Nevertheless, overexpressed Ano8, 9 and 10 show poor membrane expression. Since endogenous anoctamins are expressed at much lower levels, these anoctamins may not even accumulate at significant numbers in the plasma membrane of native cells. It is therefore entirely possible that Ano8, 9 and 10 have an intracellular rather than plasma membrane function. Currently available commercial antibodies are mostly of insufficient quality to stain endogenous anoctamins. Noteworthy, in contrast to Duran and colleagues (Duran et al., 2012), we expressed only human anoctamins and acutely activated anoctamins, by either receptor stimulation or by ionomycin. Since activation of most anoctamins was found to be transient, it is possible to overlook these currents, particularly if they are small, as those found for Ano5, 7, 8 and 9 (Figs 1, 2). In summary, our present results suggest that anoctamins form a family of Ca\(^{2+}\)-activated Cl\(^{-}\) channels, which may have their function in the plasma membrane or in intracellular compartments. In addition to

![Fig. 8. Plasma membrane localization of anoctamins.](image)
their property as Ca\(^{2+}\)-activated Cl\(^{-}\) channels, anoctamins may either have some permeability for cations, or their expression activates independent cation channels. These results may help to understand the physiological function of anoctamins and their role in disease.

**Materials and Methods**

**Cell culture, cDNAs, siRNAs and transfection**

HEK293 and HT\(_22\) cells were grown in DMEM and DMEM-F12, respectively (GIBCO, Invitrogen, USA). Cells were transfected with 10\(\mu\)g total DNA or RNA using Lipofectamine 2000 (Invitrogen) as described earlier. Successful knockdown of anoctamin was demonstrated by real time RT-PCR (Kunzelmann et al., 2011a).

**Patch clamping**

Following transfection (2–3 days) of His-tagged anoctamins or siRNA, respectively, overexpressing HEK293 or siRNA-transfected HT\(_22\) cells were identified by incubating the cells 1–2 mm with Dynabeads CD8 (Invitrogen). His-

**Measurement of intracellular Ca\(^{2+}\) concentration**

For single cell fluorescence measurements of HEK293 cells were grown on glass coverslips, mounted in a cell chamber and perfused with ringer solution at 8 ml/min at 37°C. Cell fluorescence measurements were measured continuously on an inverted microscope Axiovert S100 (Zeiss, Germany) with a Fluva 20x/0.75 objective (Zeiss, Germany) and a high speed polychromator system (VisiChrome, Visitron Systems, Germany). Cells were loaded with 2 \(\mu\)M Fura-2, AM (Molecular Probes) in ringer solution or Opti-MEM (GIBCO) under experimental conditions with 0.2% pluronic (Molecular Probes) for 1 h at 37°C. Fura-2 was excited at 340/380 nm, and the emission was recorded between 470 and 550 nm using a CCD camera (CoolSnap HQ, Visitron Systems, Germany). Control of experiment, imaging acquisition, and data analysis were performed using the software package Meta-Fluor (Universal imaging, USA) and Origin (OriginLab Corporation, USA).

**Iodide quenching**

Quenching of the intracellular fluorescence generated by the iodide-sensitive enhanced yellow fluorescence protein (YFP) was used to measure anion conductance. YFP fluorescence was excited at 490 nm using a semi-automatic Novostar plate reader (BMG-Labtech, Offenbach, Germany). I\(^{-}\) influx was induced by replacing 20 mM extracellular Cl\(^{-}\) with I\(^{-}\). Background fluorescence was subtracted and autofluorescence was negligible. Changes in fluorescence induced by I\(^{-}\) are expressed as initial rates of fluorescence decrease (arbitrary units/sec).

**Immunocytochemistry**

Transfected HEK293 cells were grown on glass coverslips and fixed for 10 min with 4% (v/v) paraformaldehyde at room temperature. Cells were incubated for 50 min with 0.1% SDS in PBS. After washing, cells were permeabilized and blocked with 2% (v/v) PBS-BSA and 0.4% (v/v) PBS Triton X-100 for 1 h and incubated overnight with primary anti-His AB (1:500, Quagen, Hilden, Germany) and anti-\(\beta\)-catenin (1:500, Sigma, Taufkirchen, Germany) at 37°C. Binding of the primary antibody was visualized by incubation with a secondary donkey anti-

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