Anoctamin 6 is an essential component of the outwardly rectifying chloride channel

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Outwardly rectifying chloride channels (ORCC, ICOR) of intermediate single-channel conductance of around 50 pS, are ubiquitously expressed, but have remained a mystery since their description more than 25 y ago. These channels have been shown to be activated on membrane excision and depolarization of the membrane voltage and by cAMP in the presence of the cystic fibrosis transmembrane conductance regulator. We show that anoctamin 6 (Ano6), a member of the recently identified family of putative Cl− channels, is the crucial component of ORCC single-channel and whole-cell currents in airway epithelial cells and Jurkat T lymphocytes. Cystic fibrosis transmembrane conductance regulator augmented ORCC produced by Ano6 in A549 airway epithelial cells. Ano6 is activated during membrane depolarization or apoptosis of Jurkat T lymphocytes and epithelial cells, and is inhibited by 5-nitro-2-(3-phenylpropylamino) benzoic acid, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, or AO1. Ano6 belongs to the basic equipment of any cell type, including colonic surface epithelial cells. It forms the essential component of ORCC and seems to have a role for cell shrinkage and programmed cell death.

Outwardly rectifying chloride (Cl−) channels (ORCC, ICOR, ORDCC) of intermediate single-channel conductance of about 50 pS are expressed abundantly (1–3). These channels are activated upon excision of the cell membrane from the intact cell and after strong depolarization (1–5). It is therefore unclear whether ORCC contributes to epithelial salt transport and whether it has a role in cystic fibrosis (1–4, 6). Subsequent reports claimed that the cystic fibrosis transmembrane conductance regulator (CFTR) and ORCC are distinct proteins with a regulatory relationship and that both channels contribute to the cAMP-dependent Cl− conductance (5, 7, 8). Similar to epithelial cells, ORCC is silent in intact lymphocytes, but it is activated after membrane excision or depolarization (9). Also, ORCC was detected in intact Jurkat lymphocytes upon Fas ligand (FasL)-induced apoptosis (10). Moreover, ORCC activated by FasL or radical oxygen species are phenotypically similar to ORCC activated by FasL did not inactivate at depolarized clamp voltages (Fig. S1D). ORCC was inhibited by NPPB, the anoctamin-blocker CaCCinh-A01 (AO1) (16), and partial removal of extracellular Cl− (Fig. 1A–C and Fig. S1A and B). Activation of Fas receptors leads to formation of ceramide, which also activated NPPB and AO1-sensitive ORCC in the present study, and which induced apoptosis in Jurkat cells (10) (Fig. 1D and Fig. S1E).

Ano6 (TMEM16F) is the prominently expressed anoctamin in Jurkat cells, apart from lower levels of Ano5 (TMEM16E) and Ano8 (TMEM16H) (Fig. S1G). We speculated that Ano6 may have a role for ORCC in Jurkat cells and therefore knocked down expression of Ano6 using three independent siRNAs (Fig. S1H). Knockdown of Ano6 completely abrogated activation of ORCC by FasL, whereas scrambled RNA had no effects (Fig. 1E–G and Fig. S2A). Moreover, siRNA knockdown of Ano5 or Ano8 did not suppress activation of ORCC (Fig. 1H and I). These results suggest that Ano6 produces the ORCC in Jurkat cells.

We further examined ORCC single-channel activity in inside/out excised membrane patches. The incidence of ORCC was low in membranes excised from control Jurkat cells (Fig. 2A). In contrast, ORCC with a single-channel conductance of around 50 pS were frequent in excised patches from cells stimulated by the Fas ligand (Fig. 2B). The single-channel conductance (Fig. 2C) and mean open probability (NPo; cf. Materials and Methods) of ORCC was larger at depolarized clamp voltages. NPo was potently inhibited by NPPB and AO1 (Fig. 2D and E). Knockdown of Ano6 by siRNA inhibited NPo of FasL-activated ORCC significantly in both cell-attached and cell-excised membrane patches, supporting the concept that Ano6 is a major component of ORCC (Fig. 2F). ORCC was activated by exposure of excised membrane patches to ceramide, and again treatment with siRNA for Ano6 reduced NPo of ceramide-activated ORCC channels (Fig. 2G and H).

Staurosporine (STS) is also known to activate ORCC and to induce apoptosis (11, 17). Similar to FasL, also STS activated ORCC whole-cell and single-channel currents in Jurkat cells and induced apoptosis (Fig. S1F). STS-activated ORCC was inhibited by NPPB and AO1 and was suppressed by siRNA-Ano6 (Fig. S3A–E). Also, cell death was reduced by Ano6 knockdown (Fig. S3F). To further validate that ORCC whole-cell currents are produced by the observed single channels, we analyzed the stationary whole-cell current noise at +50 and −50 mV clamp voltage. The variance of the steady-state current was determined,

Materials and Methods

Ano6 Causes ORCC in T Lymphocytes. Outwardly rectifying whole-cell Cl− currents were activated in Jurkat T lymphocytes after stimulation of CD95 cell death (Fas) receptors with FasL, which also induced apoptosis (Fig. 1A–D and Fig. S1C). Notably, the ORCC activated by FasL did not inactivate at depolarized clamp voltages (Fig. S1D). ORCC was inhibited by NPPB, the anoctamin-blocker CaCCinh-A01 (AO1) (16), and partial removal of extracellular Cl− (Fig. 1A–C and Fig. S1A and B). Activation of Fas receptors leads to formation of ceramide, which also activated NPPB and AO1-sensitive ORCC in the present study, and which induced apoptosis in Jurkat cells (10) (Fig. 1D and Fig. S1E).

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and the single-channel current \( i \) was determined according to \( \text{var}(I) = \langle I \rangle (1 - \langle P \rangle) \). The calculated single-channel current \( i \) was \( -1.40 \pm 0.25 \text{ pA} \) at \(-50 \text{ mV} (n = 4) \), assuming a single-channel open probability \( \langle P \rangle \) of 0.28. This compares well with \( i = -1.48 \pm 0.3 \text{ pA} (n = 5) \) measured in single-channel recordings. At \(+50 \text{ mV} \), calculated \( i \) was \( +2.90 \pm 0.11 \text{ pA} (n = 5) \) using \( \langle P \rangle \) of 0.62, which corresponds well with a measured \( i \) of \(+2.60 \pm 0.4 \text{ pA} (n = 5) \).

**Ano6 Causes ORCC in Airway Epithelial Cells.** Many studies report ORCC in epithelial cells (1–3, 18), which corresponds well to the broad expression of Ano6 in all epithelial tissues and cell lines analyzed here and in a previous report (15) (Fig. 3 A and B). Previous reports indicate a role of ORCC during apoptotic cell death, and Ano6 was reported as a Ca\(^{2+}\)-dependent phospholipid scramblase (10, 19). We analyzed expression of Ano6 in the colon epithelium, which is generated every 72–96 h. This tissue shows a high rate of apoptosis (20). We found pronounced expression of Ano6 in surface epithelial cells of mouse colon. These cells are known to move into apoptosis, whereas proliferating colonic crypt cells do not express Ano6 (Fig. 3C).

We examined activation of ORCC by STS in human alveolar epithelial (A549) and airway epithelial (9HTE) cells. In 9HTE cells, ORCC had been identified earlier (21). A549 and 9HTE cells predominantly express Ano6 (Fig. S3G) and do not present endogenous Ca\(^{2+}\)-activated Cl\(^-\) currents. STS activated whole-cell Cl\(^-\) conductances in A549 and 9HTE cells that were inhibited by NPPB, AO1, and DIDS, respectively, or by siRNA knockdown of Ano6 in 9HTE cells (Fig. 3 D–G and Fig. S2C). To further support the notion that Ano6 forms ORCC, we overexpressed Ano6 in A549 cells. This caused a NPPB-sensitive ORCC, even in the absence of STS (Fig. 4A). In excised membrane patches from Ano6-overexpressing cells, a large outwardly rectifying current that was inhibited by NPPB and that could no longer be resolved as single-channel openings was observed (Fig. 4 B and C). Furthermore, we generated an A549 cell line with a stable knockdown of Ano6 expression (A549/sh-Ano6), using short hairpin RNA constructs (Fig. 4D). Activation of

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**Fig. 1.** Stimulation of the FAS receptor activates Ano6-dependent whole-cell currents. (A and B) Whole-cell currents were measured in control Jurkat cells and after exposure to the Fas ligand (FasL; 500 ng/mL) for 2 h. Cells were voltage clamped to \(-80 \text{ mV} \) in increments of \( 10 \text{ mV} \). Outwardly rectifying whole-cell currents (ORCC) were activated by FasL and inhibited by the chloride channel blockers NPPB (N; 100 \( \mu \text{M} \)) or CaCC\(_{\text{vpt}}\)-AO1 (A; 20 \( \mu \text{M} \)). (C) Summary of whole-cell conductances measured in control Jurkat cells and after exposure to FasL. FasL-induced ORCC was completely inhibited by NPPB and AO1. (D) Current/voltage (I/V) relationship for ORCC activated by C2/C6 ceramide (20 \( \mu \text{M} \)), but not by inactive ceramide. (E and F) I/V relationships for control cells and cells exposed to FasL. Knockdown of Ano6 expression by siRNA (si-Ano6) completely abrogates activation of ORCC by FasL. (G) Summary of whole-cell conductances in FasL-stimulated Jurkat cells, which have been treated with si-Ano6 or scrambled RNA. (H and I) I/V relationships before and after FasL stimulation of Jurkat cells treated with siRNA for Ano5 or Ano8. Knockdown of Ano5 or Ano8 had no effect on activation of whole-cell currents by FasL. Mean \( \pm \text{SEM} \); n = number of experiments. *\( p < 0.05 \), paired \( t \) test; \( *p < 0.05 \), unpaired \( t \) test.

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**Fig. 2.** Ano6 produces ORCC single channels. (A and B) Single-channel activity in excised inside/out membrane patches from control Jurkat cells and cells exposed to FasL (500 ng/mL) for 2 h. The open probability \( NP_{o} \) of ORCC was larger at depolarized membrane voltages. (C) The single-channel conductances were identical for ORCC detected in patches from nonstimulated cells or cells exposed to FasL. (D) \( NP_{o} \) of ORCC in inside/out patches was largely increased after exposure to FasL and was inhibited by 100 \( \mu \text{M} \) NPPB or 20 \( \mu \text{M} \) AO1. (E) ORCC channels in inside/out patches were potently inhibited by NPPB or AO1. (F) Summary of \( NP_{o} \) measured for ORCC in cell-attached -excised patches from control Jurkat cells, cells exposed to FasL for 2 h, and after treatment with Ano6-siRNA (48 h) and FasL. (G and H) Activation of ORCC by ceramide (20 \( \mu \text{M} \)) in an excised inside/out membrane patch from a control cell, and summary of \( NP_{o} \) of ORCC in control cells and cells treated with siRNA for Ano6. Mean \( \pm \text{SEM} \); n = number of experiments. \( *p < 0.05 \), paired \( t \) test; \( *p < 0.05 \), unpaired \( t \) test.
ORCC by STS was completely suppressed in this cell line, but was regained by expression of exogenous Ano6 (Fig. 4E).

Further experiments were performed to determine the role of Ano6 for ORCC. We mutated amino acids within in the putative pore-forming domain of Ano6 (Y405F, K553A, K834A) (12) and found a significant change in halide permeability when Y405F-Ano6 was expressed in HEK293 cells, suggesting that Ano6 itself conducts anions. (Fig. 4F and G). ORCC has been reported to be activated by depolarizing voltages (1–5). We exposed both Jurkat and A549 cells to short (15–60 s) depolarizing voltages (+80 mV), which activated ORCC currents in both cell lines (Fig. 4H and I). Taken together, these results and the fact that single ORCC channels were inhibited by DIDS, NPPB, and AO1 in all three cell lines suggest that Ano6 forms a major component of ORCC (Fig. S3I).

**Activation of ORCC by CFTR Requires Ano6.** CFTR was reported to activate ORCC (5, 21, 22). To examine the effect of CFTR on Ano6 and ORCC, we made use of an A549 cell line stably expressing a tetracycline-inducible CFTR-Cherry construct (Fig. 5A).

We examined ORCC before and after induction of CFTR expression and in the absence or presence of Ano6-siRNA (Fig. 5B). Without induction of CFTR expression (−CFTR), an outwardly rectifying Cl− conductance was induced by STS that was absent after siRNA knockdown of Ano6.

ORCC activated by STS was significantly larger in cells expressing CFTR (+CFTR). Moreover, the conductance inhibited by Ano6-siRNA was significantly increased (Fig. 5F). Ano6 was a component of cAMP-activated whole-cell currents in CFTR-expressing cells because cAMP-activated whole-cell conductance was reduced after knockdown of Ano6 (Fig. 5C). Finally, after activation of CFTR by IBMX and forskolin (i.e., the ORCC blocker AO1 inhibited a larger portion of the current (Fig. 5D). Compared with the effects of AO1 in nonstimulated cells (ΔGΔAO1 = 1.72 ± 0.3 nS; n = 5), inhibition of ORCC by AO1 was clearly enhanced (ΔGΔAO1 = 12.51 ± 1.2 nS; n = 5) after blocking CFTR Cl− currents with CFTRinh-172 in I/F-stimulated cells. These experiments suggest that ORCC is augmented by cAMP in the presence of CFTR. Both ORCC and CFTR single-channel currents were detected in CFTR-expressing A549 cells (Fig. 5E and F). ORCC channels of 50 pS, but not 7-pS CFTR channels, were inhibited by AO1 (Fig. 5E). Taken together, Ano6 produces ORCC that is activated by proapoptotic stimuli, and activation
Ano6 is an essential component of ORCC. The present experiments provide reasonable evidence that Ano6 is the molecular counterpart of the abundant ORCC that appear to be part of the basic cellular equipment: (i) Ano6 is found in all cells that express ORCC (1–3, 18). (ii) Knockdown of Ano6 inhibits ORCC. (iii) Overexpression of Ano6 induces ORCC. (iv) Biophysical properties and the pharmacology of ORCC and Ano6-induced Cl⁻ currents are comparable. (v) CFTR regulates ORCC and Ano6 currents. (vi) A mutation in the putative pore-forming domain of Ano6 changes the anion permeability of ORCC. (vii) Both ORCC and Ano6 seem to have a role in apoptotic cell shrinkage and cell death (23). Excitingly, Ano6 was shown to control Ca²⁺-dependent scrambling of phospholipids, which is expected to influence a broad range of biological functions, including apoptosis (19).

We reported a small Ca²⁺-activated Cl⁻ conductance in Ano6-overexpressing cells, although Ano6 was not rapidly activated by 1 µM [Ca²⁺]ᵢ in patch clamp experiments (15). Here we did not find evidence for a Ca²⁺-dependent regulation of Ano6/ORCC currents: (i) Ca²⁺-activated Cl⁻ currents were not detected in any of the cell lines examined here. (ii) Ano1 is not expressed in any of the cell lines used here. (iii) Activation of ORCC by FasL or STS was not affected by preincubation with the Ca²⁺ chelator BAPTA-AM (Fig. 2H). (iv) Activation of ORCC in Jurkat cells was not reduced in the absence of Ca²⁺ in the pipette-filling solution (6.2 ± 1.3; n = 5 vs. 5.7 ± 0.9 nS; n = 6). Depending on the cell type, ORCC shows variable rectification, single-channel conductances, and open probabilities (1–4, 10). However, the halide permeability sequence of ORCC in the different cell lines is comparable and is SCN⁻ > I⁻ > Br⁻ > Cl⁻ > gluconate (18). Homodimerization has been shown meanwhile for Ano1 (24, 25). Although heterooligomerization has not yet been detected, we speculate that Ano6 may form variable oligomeric protein complexes in different cell types, thereby shaping ORCC in different cell types (15). Moreover, association with accessory proteins is possible and should be examined in the future.

**Regulation by CFTR and Role for Apoptotic Cell Shrinkage.** Interestingly, apoptosis of A549 cells was induced only after induction of CFTR expression, which demonstrates the proapoptotic function of CFTR (17) (Fig. 5G and H). This and other results shown here indicate a positive coupling of Ano6 and CFTR, although we were not able to detect a direct physical interaction between CFTR and A01 in coinmunoprecipitation experiments. Moreover, cell death induced by STS was attenuated by siRNA knockdown of Ano6 (Fig. 5H). This suggests a role of Ano6 in apoptotic cell death. Ano6 may control cell volume, as suggested earlier (23), and may facilitate apoptotic cell shrinkage, which is an early step during apoptosis (11). siRNA knockdown of Ano6 and other anoctamins inhibited I_Cl,swell in different cell lines, and I_Cl,swell was reduced in tissues from Ano1 null mice (23). In the present experiments, STS induced a considerable cell shrinkage, which was attenuated by the Ano6 inhibitor AO1 (Fig. S4A).

Also, regulatory volume decrease after hypotonic cell swelling was augmented in HEK293 cells overexpressing Ano6 (Fig. S4B and C). Finally, VSOR chloride currents are typically inhibited by higher extracellular ATP concentrations (26). We found a significant inhibition of Ano6/ORCC by 2 mM extracellular ATP (Fig. S4D and E). Taken together, the present results suggest that ORCC is activated by cell swelling and is formed by Ano6. The present results may therefore help to understand the functional coupling between CFTR, ORCC, and apoptotic cell shrinkage. CFTR has been shown to regulate intracellular ceramide levels and to affect apoptosis by controlling glutathione transport. It is also entirely possible that the soluble cytosolic inhibitor of ORCC described earlier, is related to the metabolism of reactive oxygen species (17, 27, 28).

**Materials and Methods.** Materials. The following materials were obtained from the indicated suppliers: C2-ceramide and C2-dihydroceramides-ceramide from Enzo Life Science; C6-ceramide from Cayman Chemical; hMEM166 siRNA from Santa Cruz Biotechnology; TMEM16H siRNA and GFP antibody from Invitrogen; hAno6 siRNA from Invitrogen and Ambion; CD95 antibody (Fas ligand, human active 18171
clone CH-11) from Millipore; and CFTR antibody from the Cystic Fibrosis Foundation (Bethesda, MD) Ano6 C-terminal antibody was synthesized by David A. Skullerud/Technologies. Other chemicals were purchased from Sigma or Calbiochem.

Cell Culture and Transfection. Jurkat cells were cultured in RPMI1640 supplemented with 10% FBS, 2 mM l-glutamine, and 1% penicillin/streptomycin. AS49 doxycycline (DOX)-inducible mCherry-FLAG-CFTR was grown in DMEM media supplemented with 10% FBS, 1% penicillin/streptomycin. Other cell lines used were cultured according to standard protocols described in ATCC. For CFTR induction, AS49 cells were treated with 1 μg/mL of DOX and 10 mM sodium butyrate for 18 h. Jurkat cell transfection was carried out by Lipofectamine LTX (Invitrogen), and AS49 cell transfection was performed using Lipofectamine 2000 (Invitrogen). Experiments were performed 48–72 h after transfection. Mutations of human Ano6 (Y405F, K553A, K834A) were generated by PCR techniques using Pfu Ultra II polymerase (Agilent Technologies). Correct sequences were verified by sequencing. Six different expression-rearranged lentiviral short hairpin RNA constructs (pGIPZ) targeting human Ano6 (ThermoFisher Scientific) were used to generate a stable Ano6 knockdown (AS49/sh-Ano6).

Patch Clamp. Jurkat cells were allowed to sit for 5 min on glass coverslips coated with 0.01% poly-L-lysine (Sigma), and AS49 cells were grown directly on glass coverslips. Patch clamp procedure has been described elsewhere (23). Membrane voltage (Vm) was clamped in steps of 10 mV, 500 ms each, from −80 to +80 mV. The product of the number of channels (N) times the open probability (Po), the mean open probability, NPo, was used as a measure for the activity of channels in a patch. NPo was calculated using the relationship (Eq. 1)

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NPo = \frac{N}{nT}
\]

with N as the maximal number of channels, n as the number of channels open, t as the time during recording when there were n channels open, and T as the total recording time.

FACS. Cells were stained with propidium iodide staining solution and an antibody against Annexin V (BD Biosciences). Cells were analyzed on a FACSCalibur with CellQuest software (BD Biosciences).

Western Blot, Ca2+ Measurement, RT-PCR, and Analysis of Cell Volume Changes. Western blot and Ca2+ measurement procedures were described in the work by Barro-Soria et al. (29). RT-PCR and analysis of cell volume was performed according to previous studies (15, 22).

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