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Calcium-activated and apoptotic phospholipid scrambling induced by Ano6 can occur independently of Ano6 ion currents

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Immune cells and platelets maintain plasma membrane phospholipid asymmetry. Upon activation, this asymmetry is disrupted by phospholipid scrambling (PS), which is a major step during activation of immune cells, hemostasis and apoptosis. Anoctamin 6 (Ano6; TMEM16F) causes chloride (Cl^-) and cation currents and is required for Ca^{2+}-dependent PS. It is defective in blood cells from patients with Scott syndrome, a rare bleeding disorder. We examined if Cl^- currents and PS are related, whether both processes are Ca^{2+} dependent, and whether Ca^{2+}-independent scrambling during intrinsic and extrinsic apoptosis is controlled by Ano6. Ca^{2+} increase by ionomycin activated Ano6 Cl^- currents and PS in normal lymphocytes, but not in B-lymphocytes from two different patients with Scott syndrome. Fas ligand (FasL) did not increase intracellular Ca^{2+}, but activated Cl^- currents in normal but not in Scott lymphocytes. Whole-cell currents were inhibited by Cl^- channel blockers and by siRNA knockdown of Ano6. In contrast, intrinsic mitochondrial apoptosis by ABT-737 did not induce Cl^- currents in lymphocytes. PS was not inhibited by blockers of Ano6 or removal of Cl^- ions. Remarkably, Ca^{2+}-independent scrambling due to extrinsic (FasL) or intrinsic (ABT-737) apoptosis was unchanged in Scott cells. We conclude that: (i) Ano6 Cl^- currents are activated by increase in cytosolic Ca^{2+}, or Ca^{2+} independent by stimulation of Fas receptors; (ii) Ca^{2+}-dependent PS induced by Ano6 does not require Cl^- currents; (iii) Ca^{2+}-independent PS does not require Ano6; (iv) Ano6 is necessary for Ca^{2+}-dependent PS, but not by increasing intracellular Ca^{2+}.

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Endogenous or overexpressed anoctamins produce chloride (Cl^-) or cation currents.[1–8] This putative family of Cl^- channels demonstrate variable Ca^{2+} sensitivity and are activated during cell swelling or by apoptotic stimuli.[9–12] Anoctamins are unique as they do not show any obvious homology to other ion channels. Ano1 contains eight predicted transmembrane helices, intracellular amino- and carboxy-terminal ends, and a pore formed by the fifth and sixth transmembrane helices containing a *p*-loop dipping back into the membrane.[1] However, a different topology has been proposed recently.[13] Anoctamins show a high degree of sequence similarity, particularly for the putative pore region.[7] We were able to demonstrate that anoctamin 6 (Ano6) produces an outwardly rectifying Cl^- channel (ORCC) in Jurkat lymphocytes and epithelial cells, which is activated during stimulation of the pro-apoptotic Fas receptor or by the pro-apoptotic compound staurosporine.[9]

Another recently identified function of Ano6 (gene *TMEM16F*) is the regulation of membrane phospholipid scrambling (PS).[14] The so-called scramblase process disturbs the asymmetric distribution of membrane phospholipids of nonstimulated cells, in which the negatively charged phosphatidylserine distributes from the inner to the outer membrane leaflet.[15] PS and hence phosphatidylserine exposure can be induced by activation with Ca^{2+}-mobilizing agonists, and by induction of the apoptotic process. We and others have shown that phosphatidylserine exposed at the surface of platelets has an essential role in the process of blood coagulation.[15,16] Recent findings indicate that two different patients with the so-called Scott syndrome, a quite rare congenital bleeding disorder, have missense mutations in the *TMEM16F* gene.[14,17] Defective function of this gene can explain why Scott platelets and other hematopoietic cells are unable to perform Ca^{2+}-dependent phosphatidylserine exposure and, hence, are impaired in supporting blood coagulation.[16] Here, we analyzed Ano6-dependent Cl^- currents in immortalized B lymphocytes from the two Scott patients with characterized *TMEM16F* mutations, and compared these with their ability to PS under conditions of Ca^{2+} activation and apoptosis.

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Abbreviations: Ano6, anoctamin 6; PS, phospholipid scrambling; FasL, Fas ligand; $Scott_{USA}$, immortalized B-lymphocytes from a US patient with Scott disease; $Scott_{UK}$, immortalized B-lymphocytes from a UK patient with Scott disease; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; TA, tannic acid; AO1, $CaCC_{inh}$-AO1; QVD, Q-VD-Oph
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Results

Expression of anoctamins in lymphocytes. The anoctamin family comprises 10 members (Ano1-10). These channels are expressed abundantly in various cell types. We performed a semi-quantitative RT-PCR analysis of the expression of anoctamins in immortalized B lymphocytes from a healthy volunteer (wt) and from two different patients with Scott disease ($Scott_{USA}$, $Scott_{UK}$).[18,19] Anoctamin expression was semi-quantified using the house keeper protein GAPDH as an internal standard (Supplementary Figures 1A,B). We detected expression of Ano9 and low levels of Ano1 in all three lymphocyte cell lines. Ano6 mRNA was detected only in control lymphocytes and cells from the $Scott_{UK}$ patient, but not in $Scott_{USA}$ lymphocytes. Accordingly, no expression of Ano6 protein was detected in $Scott_{USA}$ lymphocytes, whereas expression of Ano6 protein appears reduced in cells from the $Scott_{UK}$ patient (Supplementary Figures 1C,D).

Calcium induced Cl^- conductance in normal lymphocytes but not in lymphocytes from Scott patients. When analyzed with the patch clamp technique, we found that normal lymphocytes had a resting membrane voltage (V_m) of -59 ± 3.6 mV, whereas V_m of $Scott_{USA}$ and $Scott_{UK}$ lymphocytes were significantly hyperpolarized (-79 ± 2.8 and -75 ± 3.7 mV) (Figures 1b, d and f). Membrane voltages were obtained as reversal potentials from I/V curves and were also measured directly under current clamp, which supplied the exact same numbers. Stimulation with the Ca^{2+} ionophore ionomycin (1 μM) activated a large whole-cell current in control lymphocytes, but not in lymphocytes from $Scott_{USA}$ or $Scott_{UK}$ patients (Figures 1, 2a and b). Membrane voltage of normal lymphocytes was largely depolarized by ionomycin to -10 ± 1.6 mV, whereas it was essentially unchanged in $Scott_{USA}$ lymphocytes, and only slightly depolarized by ionomycin in $Scott_{UK}$ lymphocytes. Depending on the presence of cations, Ano6 whole-cell currents are either linearly or outwardly rectifying.[9] In lymphocytes, currents were linear in the presence of cations (Figure 1b), but were outwardly rectifying in the absence of Na^+ and K^+ (Figure 1g and h). Under these conditions, the Cl^- channel inhibitor $CaCC_{inh}$-AO1 (AO1; 20 μM)[20] or removal of bath Cl^- (5 Cl^-) largely reduced outward currents and shifted the reversal potential by almost 40 mV (Figures 1g–i). Moreover, the whole-cell current activated in normal lymphocytes by ionomycin was significantly reduced after siRNA knockout of Ano6, which also significantly suppressed Ano6 mRNA (by $40 \pm 2.3\%$; $n = 3$), as measured by quantitative real-time RT-PCR (Figure 1j). Taken together, activation of Ano6 not only generates a Ca^{2+}-activated Cl^- conductance, but also induces a nonselective cation conductance similar to Ano6 currents activated in HEK293 cells, thereby strongly depolarizing membrane voltage.[8,9] The pronounced depolarization of membrane voltage beyond the equilibrium potential for Cl^- probably corresponds to recent findings, demonstrating induction of a nonselective cation channel by Ano6.[8] Lack of Ca^{2+}-activated currents in $Scott_{UK}$ or $Scott_{USA}$ lymphocytes was not because of diminished ionomycin-induced Ca^{2+} rise, as all three cell lines showed pronounced Ca^{2+} increase upon treatment with ionomycin as measured in single-cell analysis or in suspended cells (Figures 2c–e). This is in excellent agreement with previous findings.[18]

The whole-cell current induced by ionomycin was largely due to Cl^- movement, as indicated by replacement of extracellular Cl^- with impermeable gluconate, which strongly inhibited ionomycin-activated whole-cell currents (Figures 1i and 3a). A number of typical blockers for Ca^{2+}-activated Cl^- channels were tested, such as tannic acid (TA; 20 μM) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 100 μM), AO1 (10 μM), and 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (100 μM) which all potently blocked ionomycin-activated whole-cell conductance.[20–22] A concentration of 10 μM AO1 used in the present study had a relatively moderate effect when compared with 20 μM AO1 used in Jurkat cells to inhibit Ano6.[9] However, 20 μM AO1 inhibited Ano6 in human B-lymphocytes with similar potency as in Jurkat T-lymphocytes (Figures 1g and h).[9] Cell-dependent variations of the potency of these inhibitors is well known.[21] Neither Cl^- removal nor any of the Cl^- channel blockers showed effects on whole-cell currents in $Scott_{USA}$ lymphocytes, whereas the small residual whole-cell currents in $Scott_{UK}$ lymphocytes were attenuated by the inhibitors and by Cl^- removal (Figures 3b and c). These data imply that the Ano6 mutant expressed in $Scott_{UK}$ lymphocytes provides a residual Cl^- channel activity. It is caused by a transition at the first nucleotide of intron 6, leading to an in-frame deletion of 38 amino acids in the N-terminal cytoplasmic tail. When expressed in HEK293 cells, this Ano6 mutant generated a small but significant whole-cell Cl^- current ($\Delta I = 0.82 \pm 0.13$ nA; $n = 5$) upon activation by ionomycin (1 μM). For comparison, ionomycin-activated whole-cell current in wt-Ano6-overexpressing HEK cells was 2.93 ± 0.31 nA ($n = 8$), whereas no current ($\Delta I = 0.05 \pm 0.006$ nA ($n = 5$) was activated in mock-transfected HEK293 cells.

In normal, but not in Scott lymphocytes, Ano6 whole-cell Cl^- currents could also be activated by using a patch pipette filling solution containing 100 μM Ca^{2+}. Shortly after forming a whole-cell configuration, a current was activated and the membrane voltage was depolarized. These Ca^{2+}-activated whole-cell currents were potently inhibited by replacement of extracellular Cl^- with gluconate (5 Cl^-) (Figure 3d). No changes were observed in $Scott_{USA}$ lymphocytes, whereas a small residual Cl^- current was found in $Scott_{UK}$ lymphocytes (Figure 3e). Taken together, the data indicate that whole-cell Cl^- currents stimulated in lymphocytes by high levels of intracellular Ca^{2+} are because of the activation of Ano6. These currents are completely absent in cells completely, lacking expression of Ano6 ($Scott_{USA}$), and are dramatically reduced in lymphocytes expressing mutant Ano6 ($Scott_{UK}$).

Ca^{2+}-independent activation of whole-cell Cl^- currents. We recently demonstrated activation of Ano6 currents in Jurkat T lymphocytes, by stimulation of Fas (death) receptors.[9] We therefore examined whether activation of Fas receptors also stimulates whole-cell currents in B lymphocytes. When normal lymphocytes were incubated with Fas ligand (FasL; 2 h, 0.5 μg/ml), whole-cell currents increased and membrane voltages were depolarized, similar to current activation by ionomycin (Figures 4a and b). These

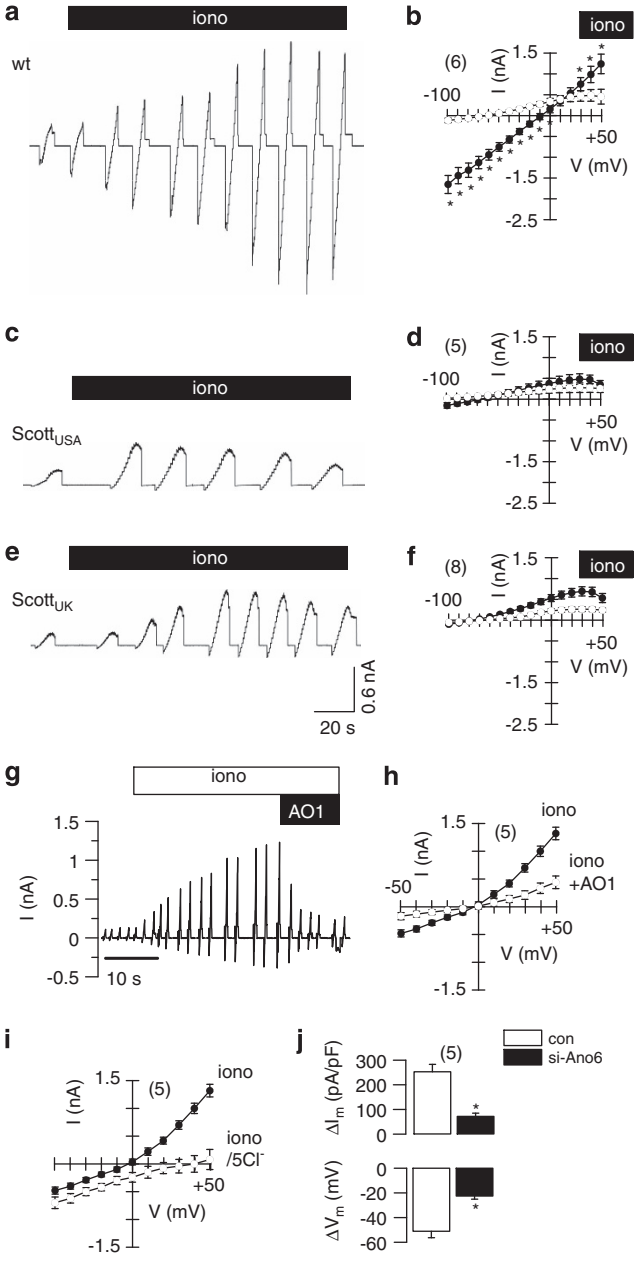

Figure 1 Ca^{2+}-dependent activation of whole-cell currents in normal but not Scott lymphocytes. Original recordings of whole-cell currents measured in normal lymphocytes (**a**) and lymphocytes from two patients with Scott disease (**c** and **e**). Cells were kept under current clamp and were voltage clamped in intervals from -100 to $+50$ mV in steps of 10 mV. Ionomycin (1 μM) activated a whole-cell current only in normal lymphocytes (**a**). Current/voltage relationships were obtained in normal (**b**) and Scott lymphocytes (**d** and **f**). Note the activation of a large whole-cell current by ionomycin (filled circles) in normal but not in Scott lymphocytes. (**g** and **h**) Activation of a whole-cell Cl^- current by ionomycin (1 μM) in normal B lymphocytes, using 145 mM $NMDG^+Cl^-$ in pipette and bath. Note the outward rectification of the whole-cell current and the pronounced inhibition by 20 μM AO1. (**i**) Inhibition of ionomycin-activated outward currents by replacement of extracellular Cl^- with gluconate (5 Cl^-). (**j**) Inhibition of ionomycin-induced whole-cell currents (current densities) and depolarization in normal B lymphocytes after knockdown of Ano6 expression with siRNA. Mean ± S.E.M. (number of cells). '*' denotes significant activation by ionomycin or effect of siRNA for Ano6 (si-Ano6); $P<0.05$; paired *t*-test

currents were inhibited by removal of extracellular Cl^- and by application of the Cl^- channel blockers TA (20 μM) and NPPB (100 μM) (Figures 4a–c). No whole-cell currents could be activated by FasL in Scott$_{USA}$ lymphocytes, whereas a small NPPB-inhibitable current was observed in Scott$_{UK}$ lymphocytes (Figures 4d and e). Notably, treatment of normal lymphocytes with siRNA for Ano6 largely reduced FasL-activated whole-cell currents ($\Delta I = 76 \pm 9.5$ pA/pF; $n = 4$), when compared with control lymphocytes ($\Delta I = 265 \pm 38$ pA/pF; $n = 8$). Because FasL did not induce any increase in intracellular Ca^{2+} (Supplementary Figure 2C), we conclude that Ano6 Cl^- currents can be activated by increase in intracellular Ca^{2+}, or in a Ca^{2+}-independent fashion by activation of the Fas receptor, similar to Jurkat T lymphocytes.[9] In Jurkat T lymphocytes, Ca^{2+} increase by stimulation of $P2Y_2$ receptors did not activate Ano6.[9] We therefore examined the effects of larger Ca^{2+} increase induced by 1 μM ionomycin. We found that also in Jurkat cells pronounced Ca^{2+} increase activates Ano6, which shows a linear I/V relationship, probably by coactivation of a non-selective cation current (Supplementary Figures 2E,F).

Stimulation of purinergic $P2X_7$ receptors can activate PS and induce apoptosis in some cell systems.[23] We therefore examined whether similar Ano6 currents are also elicited by activation of $P2X_7$ receptors in lymphocytes. Using semiquantitiative RT-PCR, we detected expression of $P2X_7$ receptors in wt and Scott lymphocytes, albeit at very low levels only, whereas Ano6 protein could not be detected by western blot (Supplementary Figure 3). Stimulation of wt lymphocytes with 3 mM ATP activated small but significant whole-cell currents, but did neither lead to a measureable increase in $[Ca^{2+}]i$ nor PS exposure (Supplementary Figures 2A,B), which is explained by the low level of $P2X_7$ receptors expression (Supplementary Figure 3). Notably, no current increase was observed in lymphocytes from patients with Scott disease.

Calcium-induced PS is independent of Cl^- conductance. As Ano6 has been reported to be crucial for Ca^{2+}-activated PS,[14] we investigated whether Ano6 Cl^- currents are required for PS . To assess PS, phosphatidylserine expression was measured after stimulation of lymphocytes with ionomycin or by exposure to FasL. Ionomycin treatment resulted in a high percentage of PS exposure in control cells, whereas PS exposure was basically abolished in Scott$_{USA}$ and Scott$_{UK}$ lymphocytes (Figure 5a). These results corresponded to the function of Ano6 as a Cl^- channel, which was also essentially absent in Scott lymphocytes. However, in contrast to the inhibition of the Cl^- channel by NPPB, TA or Cl^- replacement with gluconate, none of these maneuvers affected ionomycin-induced PS exposure in control lymphocytes (Figure 5b).

Stimulation of the extrinsic apoptosis pathway by FasL resulted in appreciable PS exposure in control cells, as well as both Scott lymphocyte cell lines, whereas Cl^- currents were only observed in wt lymphocytes (Figures 4, 5c). Although Ano6 Cl^- currents were inhibited by NPPB, TA, or by Cl^- replacement, these maneuvers did not affect FasL-induced PS exposure (Figure 5d). PS exposure in Scott$_{UK}$ and Scott$_{USA}$ lymphocytes was slightly lower compared with control cells, which corresponds to a slightly reduced

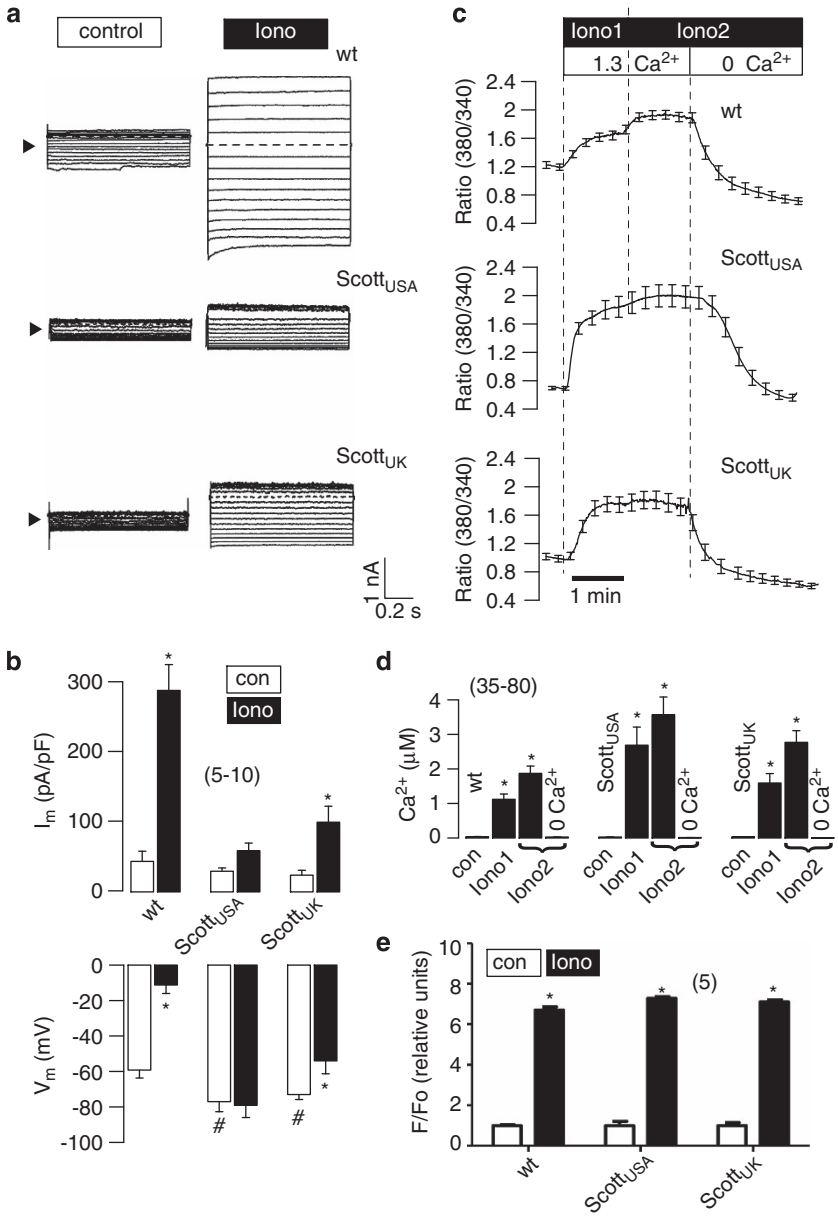

Figure 2 Lack of Ano6 in Scott lymphocytes abrogates Cl^- currents but does not affect Ca^{2+} increase. (**a**) Whole-cell overlay currents obtained in wt lymphocytes and lymphocytes from two patients with Scott disease, under control conditions and after stimulation with ionomycin (1 μM). Cells were voltage clamped from -100 to $+50$ mV in steps of 10 mV. Arrowhead indicates currents at clamp voltage of 0 mV. (**b**) Summary of whole-cell current densities and membrane voltages under control conditions and after stimulation with ionomycin (filled bars). (**c**) Continuous recordings of 380/340 ratios (Fura-2) and effects of 1 or 2 μM ionomycin (Iono1, Iono2), and Ca^{2+} removal in wt and Scott lymphocytes. (**d**) Summary of $[Ca^{2+}]$i upon application of 1 or 2 μM ionomycin. (**e**) Effects of 10 μM ionomycin on $[Ca^{2+}]$i measured in cells in suspensions (Fluo-4). Mean ± S.E.M. (number of cells). '*' denotes significant increase when compared with control; $P<0.05$; paired *t*-test

activation of caspase-3 in these cells (Figure 5e). This was confirmed by pre-treatment with the pan-caspase inhibitor, Q-VD-Oph (QVD), which completely inhibited FasL-induced PS exposure in control and Scott lymphocytes (Figure 5c). Jointly, these data indicate that FasL-induced PS is not dependent on functional Ano6.

Role of Ano6 for intrinsic apoptosis. Activation of intrinsic (mitochondrial) apoptosis is potently induced by the compound ABT-737, which inhibits B-cell lymphoma 2 and thus directly promotes Bax/Bak pore formation and consecutive release of cytochrome *c*.[24] Although ABT-737 induced a small $[Ca^{2+}]$i rise in lymphocytes (Supplementary Figure 2C), no whole-cell Cl^- currents were activated (Supplementary Figure 2D). Nevertheless, ABT-737 induced high levels of PS exposure, which were identical in Scott$_{UK}$ and Scott$_{USA}$ cells (Supplementary Figure 2E). Together, these data indicate that Ano6 Cl^- currents are activated in normal lymphocytes by elevation of $[Ca^{2+}]$i or by stimulation of extrinsic apoptosis via Fas receptors, but not during

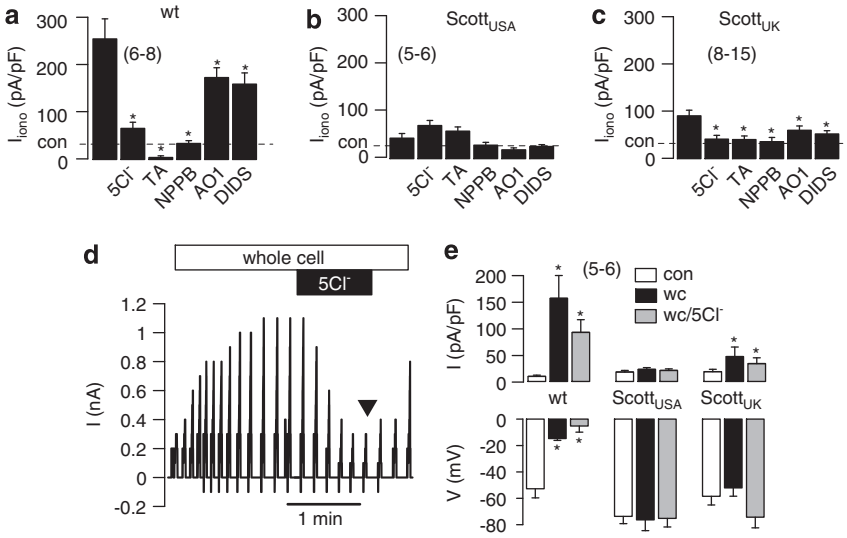

Figure 3 Ca^{2+} activates Ano6 Cl^- conductance in normal but not Scott lymphocytes. (**a–c**) Ionomycin-activated whole-cell currents in normal and Scott lymphocytes. Inhibition of currents by replacement of extracellular Cl^- with impermeable gluconate (except of 5 mM; 5 Cl^-), or by application of tannic acid (TA; 20 μM), NPPB (100 μM), $CaCC_{inh}$-AO1 (AO1; 10 μM), or 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (100 μM). Dashed lines indicate currents before stimulation with ionomycin (control). No currents were activated in $Scott_{USA}$ lymphocytes (**b**), whereas currents were largely reduced in $Scott_{UK}$ lymphocytes (**c**). (**d**) Activation of Ano6 currents by high (100 μM) pipette [Ca^{2+}] in a control lymphocyte and inhibition by removal of extracellular Cl^- (5 Cl^-). The black arrowhead in **d** indicates the time point that was chosen to read currents for generation of the summary bars shown in **a**, **b**, **c** and **e**. (**e**) Summary of current densities, indicating activation of Ano6 by pipette Ca^{2+} (black bars) and inhibition by low Cl^-. Mean ± S.E.M. (number of cells). '*' denotes significant difference compared with control; $P<0.05$; paired *t*-test

mitochondrial apoptosis. PS scrambling activated directly by [Ca^{2+}]i, extrinsic or intrinsic apoptosis does not require Ano6 Cl^- currents.

Discussion

Cl^- currents and scramblase activity by Ano6 are not directly linked. It was recently shown that Ano6 is an essential component of ORCC in Jurkat T lymphocytes that is activated by Fas receptor stimulation.[9] ORCC single channels were also observed in membrane patches excised from Jurkat cells.[9,25] Once excised into a bath solution containing high (1 mM) extracellular Ca^{2+}, the channel remained active and was only weakly Ca^{2+} dependent. It was suggested earlier that very high (cytosolic) Ca^{2+} or artificial clamp voltage may activate ORCC irreversibly.[26] The present data suggest that strong increase in intracellular Ca^{2+} by ionomycin in Jurkat cells also activates a whole-cell Cl^- current, along with a nonselective cation current (Supplementary Figures 2E,F). Although Ca^{2+} increase through receptor stimulation was insufficient to activate Ano6 in HEK293 cells and Jurkat lymphocytes,[4,9] larger increases in [Ca^{2+}]i such as those induced by ionomycin are able to activate Ano6 (Supplementary Figures 2E,F). Taken together, Ano6 can be activated by strong increase in intracellular Ca^{2+}, as well as in a Ca^{2+}-independent fashion by FasL in both Jurkat T-lymphocytes as well B-lymphocytes. How FasL activates Ano6 is currently unclear. A recent paper describes release of ATP and activation of $P2X_7$ receptors by stimulation of the Fas receptor, which would resemble one possibility how Fas is connected to activation of Ano6.[27] However, we have preliminary data indicating that preincubation (30 min) of lymphocytes with 20 μM of the broad caspase inhibitor QVD completely abolished activation of Ano6 by FasL ($\Delta I = 380 \pm 42$ pA/pF *versus* $\Delta I = 49 \pm 9$ pA/pF; $n = 7$). Thus, it may be the Fas-induced caspase itself that activates Ano6.

A recent paper reports Ano6 as a Ca^{2+}-activated nonselective cation channel.[8] The paper does not report Ca^{2+}-activated Cl^- currents related to Ano6. However, apart from our laboratory,[4] other teams also reported Ano6 as a Cl^- channel.[12,28] Similar to previous reports,[29–31] we also demonstrate in the present study that Ano6 is inducing a dominating Cl^- current when activated by high cytosolic Ca^{2+} concentrations, and that this Cl^- current is paralleled by activation of a nonselective cation current. It remains to be demonstrated whether Ano6 is a rather nonselective ion channel, or Ano6 interacts with other ion channel proteins (which might be differentially expressed in different cell types) to generate both Cl^- and cation currents.

We reported recently that expression of Ano6 in HEK293 cells and activation by ionomycin not only activates a Cl^-, but also a nonselective current.[4] This Ca^{2+}-activated nonselective cation might be related to the Ca^{2+}-activated cation current observed recently in other Ano6-expressing cells.[8] Coactivation of this cation conductance leads to strong depolarization during stimulation with ionomycin to a linear I/V curve, and to attenuated depolarization because of removal of extracellular Cl^- (Figures 1b, 3e). In contrast, when a 145 mM $NMDG^+Cl^-$ solution was used in the patch pipette and in the bath, the reversal potential of the Ca^{2+}-activated Cl^- current was zero, and I/V curves were outwardly rectifying. The reversal potential was shifted by the removal of bath Cl^-, following the Nernst equilibrium potential for Cl^- (Figures 1g–i). Thus Ano6 produces a Ca^{2+}-activated Cl^- current along with a smaller nonselective cation conductance.

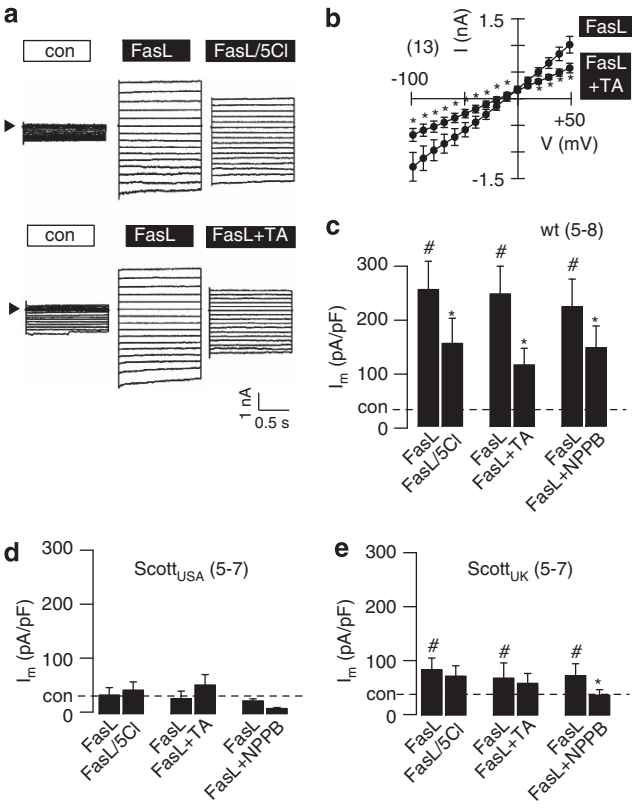

Figure 4 Activation Ano6 Cl^- currents by activation of Fas receptors. (**a**) Whole-cell current overlays obtained in normal lymphocytes before and after incubation with FasL (0.5 μg/ml; 2 h), and inhibition by Cl^- replacement (5 Cl^-) and tannic acid (TA; 20 μM). Cells were voltage clamped from -100 to $+50$ mV in steps of 10 mV. Arrowhead indicates currents at clamp voltage of 0 mV. (**b**) Current/voltage relationship of the FasL-activated whole-cell current and effect of TA. (**c**–**e**) Calculated whole-cell conductances in normal, Scott$_{USA}$, and Scott$_{UK}$ lymphocytes and effects of 5 Cl^-, TA and NPPB. Dashed line indicates conductance before incubation with FasL (control). Mean ± S.E.M. (number of cells). '*' denotes significant inhibition; $P<0.05$; paired *t*-test. '#' denotes significant activation by FasL; $P<0.05$; unpaired *t*-test

More recently, defective Ano6 has been found to be the cause for the rare bleeding disorder, Scott syndrome, which is characterized by defective Ca^{2+}-activated PS.[14,17] The present report, as well as results obtained in other cell types, point to abundant expression of this membrane protein.[9,14,32,33] In the present paper, we have studied how Cl^- channel function in healthy control and Scott syndrome lymphocytes relates to the PS process. The results indicate that Ano6 is implicated in both Ca^{2+}-activated Cl^- channel activity and PS, but only the former function was sensitive to pharmacological inhibition. Thus, the ionomycin-induced Cl^- conductance was potently suppressed by TA or NPPB, as well as by Cl^- exclusion. In contrast, these interventions did not affect ionomycin-induced PS exposure. In other words, the Scott syndrome phenotype could not be reproduced by inhibition of the Ano6-mediated Cl^- currents. This made us conclude that the Ca^{2+}-activated PS and Cl^- channel activity by Ano6 are not functionally linked.

Abrogated Cl^- conductance and scramblase activity by mutations of Ano6. An interesting observation was that ionomycin still evoked a small but significant Cl^- current and slightly depolarized the membrane voltage in Scott$_{UK}$ lymphocytes. These cells are compound heterozygous for two Ano6 mutations: (i) a transition at the first nucleotide of intron 6, leading to an in-frame deletion of 38 amino acids in the N-terminal cytoplasmic tail and (ii) a single-nucleotide insertion in exon 11, predicting a frameshift and premature termination of translation at codon 411.[17] In contrast, Scott$_{USA}$ lymphocytes are homozygous for one Ano6 mutation (IVS12–1G3T), causing exon 13 skipping, frameshift, and premature termination of translation,[14] but did not show any residual Cl^- current. The Scott$_{USA}$ mutant cells do not express any Ano6 protein and completely lack of Cl^- channel function, whereas the Scott$_{UK}$ cells retained residual Cl^- channel activity. Overexpression of this Scott$_{UK}$-specific mutant in HEK293 cells suggests a residual Cl^- channel function. In contrast, Ca^{2+}-induced PS activity was abrogated in both the Scott$_{UK}$ and Scott$_{USA}$ lymphocytes. Together, this suggests that the in-frame deletion mutation of Scott$_{UK}$ results in a protein showing limited Cl^- conductance and abolished scramblase activity, although other Ano6-independent mechanisms cannot be excluded.

Role of Ano6 for extrinsic and intrinsic apoptosis. Stimulation of the extrinsic apoptosis pathway by FasL activated both Cl^- channels and PS exposure in the control lymphocytes. No Cl^- currents were activated by FasL in both Scott lymphocyte cell lines, however, Scott cells still showed Fas receptor-induced PS exposure, which could be prevented by caspase inhibition and hence was apoptosis-induced. Similarly, direct stimulation of Bax/Bak-dependent apoptosis with ABT-737 induced normal PS exposure in the Scott lymphocytes without activating Cl^- currents. These data clearly point to a unique function of Ano6 as Cl^-/nonselective cation channel, but a redundant role of Ano6 in the regulation of apoptotic PS exposure.

We conclude that (i) Ano6 primarily induces a Cl^- conductance along with a smaller nonselective cation conductance that is activated either Ca^{2+} dependently (ionomycin) or Ca^{2+} independently (Fas receptor), but not during mitochondrial (intrinsic) apoptosis. (ii) Ca^{2+}-dependent PS induced by Ano6 occurs independent of Ano6 Cl^- conductance and (iii) Ca^{2+}-independent PS does not require Ano6. Taking into account the recent proposal that Ano6 in lymphocytes induces a Ca^{2+} conductance,[8] these findings suggest that the Scott protein Ano6[8] acts as a Ca^{2+}-dependent channel for both cations and anions, or regulates these conductances, along with activation of Ca^{2+}-dependent in lymphocytes.

Material and Methods

Cell culture. EBV-transformed B lymphoblast cell lines from control subjects and Scott$_{UK}$ and Scott$_{USA}$ patients have been described before.[18,19] The lymphocytes were grown in RPMI 1640 medium (GIBCO, 52140; Darmstadt, Germany) supplemented with 10% fetal calf serum (GIBCO) and penicillin/streptomycin (GIBCO). For patch clamping, cells were fixed on polylysine-coated coverslips.

Patch clamping. Coverslips were mounted in a heated chamber on the stage of an inverted microscope (IM35, Zeiss, Munich, Germany) and kept at 37 °C. Cells were perfused continuously with Ringer solution (in mM: NaCl 145, KH_2PO_4

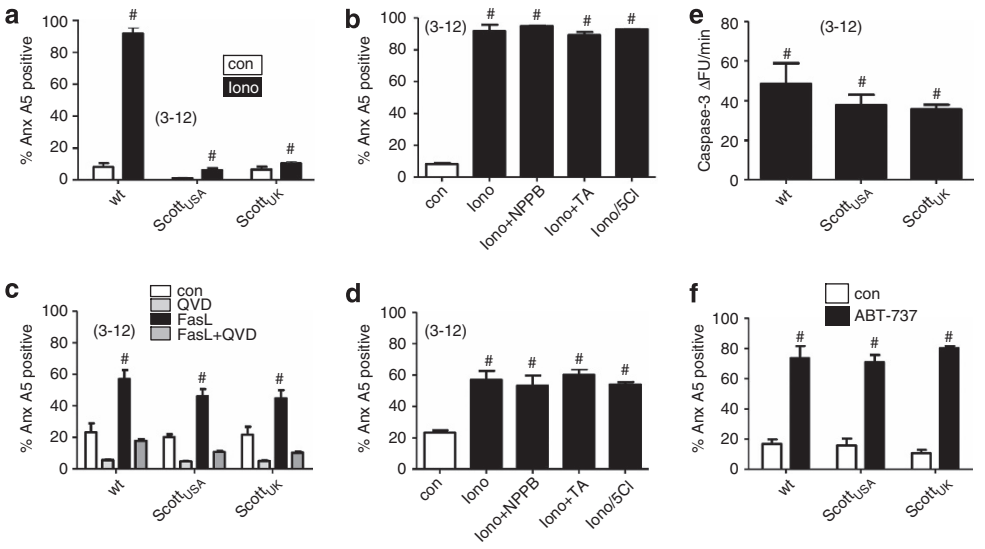

Figure 5 Phospholipid scrambling by ionomycin but not by FasL is impaired in Scott lymphocytes. (**a**) Percentage of phosphatidylserine (PS)-exposing lymphocytes after 10 min stimulation with 10 μM ionomycin in presence of 1 mM $CaCl_2$ for normal and Scott lymphocytes as measured after labeling with annexin A5 for 5 min. (**b**) Effect of NPPB (100 μM), TA (50 μM) and replacement of Cl^- by gluconate on ionomycin-induced PS exposure in healthy control lymphocytes. (**c**) Percentage PS-exposing lymphocytes after 4 h incubation with 0.5 μg/ml FasL in presence of 1 mM $CaCl_2$ for control and Scott lymphocytes in presence or absence of 20 μM Q-VD-Oph. (**d**) No effects of NPPB (100 μM), TA (50 μM) and Cl^- replacement on FasL-induced PS exposure in healthy control lymphocytes were observed. (**e**) Caspase-3 activity of control and Scott lymphocytes after 4 h stimulation with FasL as measured by a caspase activity assay. (**f**) Percentage of PS-exposing lymphocytes after incubation with 50 μM ABT-737. Mean ± S.D. ($n = 3$–12). '#' denotes significant activation of PS exposure or caspase; $P < 0.05$; unpaired *t*-test

0.4, K_2HPO_4 1.6, D-glucose 5, $MgCl_2$ 1, Ca-gluconate 1.3, pH 7.4). For fast whole-cell patch clamping, micropipettes were filled with intracellular like solution (in mM: KCl 30, K-gluconate 95, NaH_2PO_4 1.2, Na_2HPO_4 4.8, EGTA 1, Ca-gluconate 0.758, $MgCl_2$ 1.034, D-glucose 5, ATP 3, pH 7.2) and had an input resistance of 2–4 MΩ. Changes in membrane voltage were recorded in the current clamp mode. Experiments were conducted as described earlier.[34]

Measurement of intracellular Ca^{2+} concentration. Cytosolic Ca^{2+} was measured in a suspension of single cells. (i) Suspended cells were loaded with 0.5 μM Fluo-4 AM in the presence of 4 μg/ml pluronic (Molecular Probes, Darmstadt, Germany). After a washing step, cells were resuspended (3×10^5/ml) in Hepes buffer, pH 7.45 (in mM: NaCl 136, Hepes 10, KCl 2.7, $MgCl_2$ 2, glucose, and 0.1% BSA 0.1%). Agonist-induced changes in Fluo-4 fluorescence (F) were determined by flow cytometry (BD Accuri, Breda, Netherlands). Rises in Ca^{2+} are expressed as pseudo-ratio F/F_0, with F_0 representing the fluorescence before stimulation.[35] (ii) Single-cell Ca^{2+} measurements were performed with continuously perfused immobilized lymphocytes at 37 °C, using an inverted Axiovert S100 (Zeiss) microscope and a high-speed polychromator system (VisiChrome, Visitron Systems, Puchheim, Germany). Cells were loaded with 2 μM Fura-2 and AM (Molecular Probes) with 0.2% pluronic for 1 h at 37 °C. Experiments were conducted as described previously.[36]

Phospholipid scrambling (phosphatidylserine exposure). Lymphocytes were washed in Hepes buffer, pH 7.45 and diluted to a concentration of 3×10^5/ml. The cells were stimulated as indicated with ionomycin (Calbiochem, Darmstadt, Germany), FasL (Millipore, Darmstadt, Germany) or ABT-737 (Abbott Laboratories, Wiesbaden, Germany or Active Biochem (Maplewood, NJ, USA)) in the presence of 1–2.5 mM $CaCl_2$. Pan-caspase inhibitor QVD (Millipore) was given 10 min prior stimulation. Surface exposure of phosphatidylserine was assessed after 5 min labeling with FITC- or Alexa647-annexin A5 (Invitrogen, Darmstadt, Germany) by flow cytometry (BD Accuri). A gating was set to exclude apoptotic and dead cells. Propidium iodide labeling revealed that the latter cells appeared as a different population in forward/side scatter plots. Five thousand viable cells were analyzed per experiment.

Semi-quantitative RT-PCR. Total RNA was isolated from B lymphocytes, and reverse transcribed using a random primer and M-MLV reverse transcriptase (Promega, Mannheim, Germany). The RT-PCR reaction contained sense and antisense primers for anoctamins or GAPDH (0.5 μM; Supplementary Table 1), 1 μl cDNA and GoTaq polymerase (Promega). After 2 min at 95 °C, cDNA was amplified in 30 cycles for 30 s at 95 °C, 30 s at 56 °C and 1 min at 72 °C, and visualized by loading on ethidium bromide-containing agarose gels.

Western blotting. Protein was isolated from lymphocytes in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 100 mM DTT, 0.2% Triton X-100, 5 U/ml of benzonase, and 1% protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Protein was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare, Munich, Germany) using semidry transfer (Bio-Rad, Munich, Germany). Membranes were incubated with anti-Ano6 mAb (1 : 5000; Santa Cruz Biotechnology, Heidelberg, Germany) and proteins were visualized using a horseradish peroxidase-conjugated secondary antibody (dilution 1 : 10.000) and ECL detection kit (GE Healthcare).

Caspase-3 activity assay. Lymphocytes were activated in the presence or absence of QVD, as indicated. Cells were centrifuged at 2300 × *g*, and pellets were resolved in lysis buffer. Samples in lysis buffer were mixed 1 : 1 with 50 μl caspase-3 substrate mix, consisting of 9.8 μM DTT and 49 μM Ac-DEVD-AFC (Invitrogen). Fluorescence was measured in time using a Spectramax M2 reader. Caspase-3 activity was determined as changes in fluorescence units per min, relative to unstimulated samples.

Conflict of Interest

The authors declare no conflict of interest.

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