Ano1 as a regulator of proliferation

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MATERIALS AND METHODS

Control of the Cell Cycle is Critical to Most Aspects of Biology. Many organs such as the skin and mucosa are constantly turning over; others, such as the liver, proliferate in response to injury (17). Disruption of the tightly controlled cell cycle can lead to uncontrolled proliferation and tumor formation. Proliferation is a complex cellular process relying on molecular events of the cell cycle and may play a role in tumorigenesis. These data led us to conclude that Ano1 regulates proliferation at the G1/S transition of the cell cycle and may play a role in tumorigenesis.

TMEM16A; chloride channels; interstitial cells of Cajal; cell cycle; intestine

Ano1 as a regulator of proliferation. Am J Physiol Gastrointest Liver Physiol 301: G1044–G1051, 2011. First published September 22, 2011; doi:10.1152/ajpgi.00196.2011.—Ano1 is a recently discovered Ca2+-activated Cl− channel expressed on interstitial cells of Cajal (ICC) that has been implicated in slow-wave activity in the gut. However, Ano1 is expressed on all classes of ICC, even those that do not contribute to generation of the slow wave, suggesting that Ano1 may have an alternate function in these cells. Ano1 is also highly expressed in gastrointestinal stromal tumors. Mice lacking Ano1 had fewer proliferating ICC in whole mount preparations and in culture, raising the possibility that Ano1 is involved in proliferation. Cl− channel blockers decreased proliferation in cells expressing Ano1, including primary cultures of ICC and in the pancreatic cancer-derived cell line, CFPAC-1. Cl− channel blockers had a reduced effect on Ano1(−/−) cultures, confirming that the blockers are acting on Ano1. Ki67 immunoreactivity, 5-ethyl-2′-deoxyuridine incorporation, and cell-cycle analysis of cells grown in low-CI− media showed fewer proliferating cells than in cultures grown in regular medium. We confirmed that mice lacking Ano1 had less phosphorylated retinoblastoma protein compared with controls. These data led us to conclude that Ano1 regulates proliferation at the G1/S transition of the cell cycle and may play a role in tumorigenesis.

A potential role for Ano1 in proliferation is suggested by the high expression of the channel in gastrointestinal stromal tumors (GISTs). Ano1 was first discovered in GISTs and given the initial name of DOG1 (discovered on GISTs). Ano1 was described as being ubiquitously expressed on all GISTs (33) although a low percentage of Ano1-negative GISTs has been reported (21). The expression of Ano1 on all classes of ICC, including those that do not generate slow waves, the expression of Ano1 in GISTs, and the known role Cl− plays in regulating cell cycle suggest that Ano1 could be involved in the proliferation of ICC. The aim of this study was to investigate the role of Ano1-mediated Cl− currents in cellular proliferation using ICC primary cultures from mice and CFPAC-1 cells, a human pancreatic cell line that expresses Ano1.

Materials and Methods

Animals. BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Postnatal day (PND) 0–4 mice were killed by CO2 inhalation and cervical dislocation. Ano1(−/−) mice were obtained by breeding mice heterozygous for a targeted disruption of the Ano1 gene (23). The mice were maintained and the experiments were performed with approval from the Institutional Animal Care and Use Committee of the Mayo Clinic.

Genotyping. Ano1(−/−) mice were rapidly identified by staining the trachea with 0.03% Alcian blue in 25% acetic acid/ethanol, and PCR genotyping was performed to confirm all genotypes. DNA was extracted from tail samples using the Easy-DNA Isolation Kit (Invitrogen, Carlsbad, CA). A sample (50–200 ng) of DNA was used per 25-μl reaction. Ano1(+/+) primers are as follows and cycles are as follows: forward, 5′-CCTA TGAC TGCC AGGG ACGC CC-3′,
reverse, 5'-TGTT CCTG TCCC AGTC GG-3'; 94°C for 5 min, 38 times at 94°C for 30 s, 61°C for 30 s, 72°C for 45 s, 72°C for 7 min. Ano1(+/−) primers and cycles are as follows: forward, 5'-GGAG TAGA AGGT GCCG CGAA G3'- 94°C for 5 min, 94°C for 30 s, 57°C for 30 s, 72°C for 45 s for 38 cycles, 72°C for 7 min.

Cell cultures. Primary cultures containing ICC were obtained by enzymatic dissociation of the small intestine of PND 0–4 mice as previously described (35) with the following exceptions. Cells were resuspended and cultured in M199 media without phenol red (Invitrogen) supplemented with 1% antibiotic-antimycotic (Invitrogen). PND 0 cultures represent enzymatic dissociation of 1 small intestine, with 10^6 cells plated per condition. For Ano1 cultures treated with broad-spectrum chloride channel blockers, three small intestines from 120 mM Cl−/H11001 littermates (controls). Ano1(+/−) and Ano1(+/−) littersmates (controls). Cl− channel blockers were added 1 h after plating and remained in the cultures until fixation 46 h later.

CFPAC-1 cells, a human pancreatic cell line that expresses Ano1, were also used to investigate the role of Cl− fluxes in cell proliferation. Cells were grown in Iscove’s Modified Dulbecco’s Media (ATCC) supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin.

Chemicals. Three structurally different chloride channel blockers were used in our experiments. 4,4’-Disothioyanostilbene-2,2’-disulfonic acid disodium salt (DIDS), tamoxifen, and niflumic acid (all from Sigma) were used at final concentrations between 1 μM and 10 μM. Stock solutions (50–100 mM) were made in DMSO (Sigma) and stored at −20°C. Chemicals were diluted fresh in water on the day of the experiment. DMSO was diluted 1:10 in water and used at a final concentration of 1:1,000 for the vehicle control.

Low-chloride media. Custom M199 media without NaCl, CaCl2, KCl, and phenol red were ordered from Invitrogen. Cl− was supplemented to a final concentration of 12 mM and 40 mM using NaCl. The remaining ionic salts were replaced with Ca(NO3)2 (Sigma), NaNO3, and KNO3 (both from Fisher Scientific) to maintain osmolality. Commercial M199 medium without phenol red has a concentration of 120 mM Cl−. Cells were allowed to attach for 24–48 h in normal growth media before the Cl− concentration was altered.

Immunohistochemistry. Immunohistochemistry was done as previously described (35). In brief, proliferating cells were identified by either immunoreactivity for the nuclear antigen, Ki67 (Novus and Abcam), or by detection of incorporated 5-ethyl-2’-deoxyuridine (EdU) using the “Click-iT” technology (Invitrogen). Nuclei were counterstained with 4’,6-diamidino-2-phenylindole dilactate (DAPI). ICC were identified by immunoreactivity for Kit using the antibody ACK2 (eBioscience) and counted using a ×20 objective from 35 fields on two separate coverslips per condition.

ICC network volume determination. ICC network volumes were determined from confocal images of whole mount preparations of small intestine tunica muscularis of PND 0 Ano1(+/−) and Ano1(+/−) mice. Tissues were labeled for Kit and Ki67 and counterstained with DAPI (for complete methods, see Ref. 31). Four image stacks of the same ICC-MY volumes were not significantly different in the PND 0 mice compared with controls [control, 36%, 33–38; Ano1(+/−), 31%, 28–40, median, IQR, n = 5 Ano1(+/−), P = 0.4, Mann Whitney test, Fig. 1D].

Ano1(+/−) cultures have fewer proliferating ICC than Ano1 control cultures. We determined proliferation of ICC in primary cultures next. Cultures from Ano1(+/−) and Ano1(+/−) mice had fewer proliferating ICC than Ano1(+/−) cultures [Ano1(+/−), 24.9 ± 3.8; Ano1(+/−), 19.6 ± 3.6; Ano1(+/−), 15.0 ± 3.5; Ki67-positive ICC, mean ± SE, n = 4, P < 0.05, repeated-measures ANOVA with Newman-Keuls multiple-comparisons posttest, Fig. 2].

Cl− channel blockers decrease proliferation in culture. The above results suggest that Ano1 levels in ICC play a role in proliferation of ICC. To determine whether the effects of Ano1 were through its role as a Cl− channel, we used Cl− channel blockers on ICC primary cultures. Proliferation of ICC was significantly lower in cultures of ICC from BALB/c mice following treatment with each of three different Cl− channel inhibitors (vehicle, 14.1 ± 0.4; 10 μM DIDS, 6.4 ± 0.5; 10 μM niflumic acid, 6.2 ± 2.6; 1 μM tamoxifen, 6.1 ± 0.5; % EdU-positive ICC, mean ± SE, n = 4–6, P < 0.01, one-way ANOVA with Newman-Keuls multiple-comparisons posttest, Fig. 3A).

Similarly, CFPAC-1 cells, a human pancreatic cancer cell line, also had fewer proliferating cells when treated with chloride channel blockers (vehicle, 84.2 ± 1.12; 10 μM DIDS, 48.5 ± 7.5; 10 μM niflumic acid, 57.0 ± 2.0; 10 μM tamoxifen, 36.8 ± 11.5; % EdU-positive cells, mean ± SE, n = 4, P < 0.05, one-way ANOVA with Newman Keuls multiple-comparisons posttest, Fig. 3B).
There are no specific blockers for Ano1, and, although DIDS, niflumic acid, and tamoxifen block Cl⁻ channels, they also have other effects (5, 28). Therefore, to confirm that the effect of Cl⁻ channel blockers on proliferation was through inhibition of Ano1, we cultured ICC from Ano1⁻/⁻ mice and littermate controls with Cl⁻ channel blockers. As in BALB/c mice and CFPAC-1 cells, the effect of Cl⁻ channel blockers on proliferating ICC from control mice was a significant decrease (vehicle, 16.2 ± 2.4; 10 μM DIDS, 7.7 ± 0.7; 10 μM niflumic acid, 7.7 ± 2.8; 10 μM tamoxifen, 8.3 ± 2.4; % Ki67-positive cells, mean ± SE, n = 4, P < 0.05, repeated-measures ANOVA with Newman-Keuls multiple-comparisons posttest, Fig. 4A). These effects were not observed in cultures from Ano1⁻/⁻ mice (vehicle, 10.0 ± 1.6; 10 μM DIDS, 7.1 ± 0.9; 10 μM niflumic acid, 8.1 ± 1.8; 10 μM tamoxifen, 12.2 ± 1.2; % Ki67-positive cells, mean ± SE, n = 4, P > 0.05, repeated-measures ANOVA with Newman-Keuls multiple-comparisons posttest, Fig. 4B). Proliferation in ICC from Ano1⁻/⁻ cultures was significantly more affected by all the Cl⁻ channel blockers than was proliferation in ICC from Ano1⁻/⁻ mice (10 μM DIDS, 8.57 ± 2.90; 1 μM tamoxifen, 7.91 ± 2.16; 10 μM niflumic acid, 8.52 ± 1.94; change with respect to vehicle, n = 4, P < 0.05, two-way ANOVA with Bonferroni posttest).

![Figure 1](http://ajpgi.physiology.org/) Small intestine of Ano1⁻/⁻ mice have fewer proliferating interstitial cells of Cajal (ICC). A: projections of confocal image stacks showing proliferating ICC (arrows) in whole mounts from postnatal day (PND) 0 Ano1⁻/⁻ and Ano1⁻/⁻ mice. Nuclei of dividing cells were identified by Ki67 immunoreactivity (green). ICC were identified by immunoreactivity for Kit (red). B: significantly fewer dividing ICC were detected in whole mounts from Ano1⁻/⁻ mice [**P < 0.01, repeated-measures ANOVA with Newman-Keuls posttest, n = 7]. C: there was no difference in the Kit-positive volume between the two groups of mice (P > 0.05, unpaired t-test, n = 6). D: proliferation of cells in the circular muscle layer that do not express Ano1 was unaltered in Ano1⁻/⁻ mice compared with controls [median, IQR, n = 7 control, n = 5 Ano1⁻/⁻, P = 0.4, Mann Whitney test].

![Figure 2](http://ajpgi.physiology.org/) Ano1⁻/⁻ cultures have fewer proliferating ICC. A: representative images of ICC in culture from PND 0 Ano1 mice. Arrows show proliferating ICC as identified by colocalization of Kit (red) and Ki67 immunoreactivity (green). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dilactate (blue). B: there were fewer proliferating ICC in cultures from Ano1⁻/⁻ and Ano1⁻/⁻ mice than from Ano1⁺/⁺ littermates (**P < 0.05, repeated-measures ANOVA with Newman-Keuls posttest, n = 4).
confirming that the blockers were acting on Ano1 and that Ano1 is a mediator of proliferation.

**Low-chloride media reduces proliferation in both ICC cultures and CFPAC-1 cells.** To further determine the effect of Cl⁻ entry on proliferation, we measured proliferation in response to various Cl⁻ concentrations in the medium. Cl⁻ concentration was modulated by replacing Cl⁻ with NO₃⁻ while maintaining the osmolality of the medium. Fewer proliferating ICC cells were detected when Cl⁻ in the medium was reduced to 12 mM (120 mM, 53.6 ± 2.0; 40 mM, 61.2 ± 4.7; 12 mM, 63.6 ± 2.0; % of cells in G₁, mean ± SE, n = 3, P < 0.05, repeated-measures ANOVA with Newman–Keuls multiple-comparisons posttest, Fig. 5A). There were also a significant decrease in the proportion of cells in S-phase (120 mM, 36.1 ± 1.0; 40 mM, 27.6 ± 4.3; 12 mM, 23.4 ± 3.3; % of cells in S-phase, means ± SE, n = 3, P < 0.05, repeated-measures ANOVA with Newman–Keuls multiple-comparisons posttest, Fig. 6B).

Phosphorylated Rb is decreased in mice lacking Ano1. Because there was an increase in the proportion of cells in G₁ when grown in low-chloride media, we used the hyperphosphorylation of Rb to study the G₁/S transition. If Ano1 is important for entry into S-phase, then Ano1⁻⁻⁻ mice should have less phosphorylated (serine 780) Rb. Indeed, we found that Ano1⁻⁻⁻ mice had a lower ratio of phosphorylated (serine 780) Rb to total Rb compared with littermate controls (Ano1⁺⁺⁺, 5.98 ± 0.784; Ano1⁻⁻⁻, 3.60 ± 0.491; means ± SE, n = 4, P < 0.05, 2-way ANOVA with Bonferroni posttest, Fig. 4A).

**Proportion of cells in G₁ is increased when cultured in low-chloride media.** Cell-cycle analysis in the CFPAC-1 cells revealed a greater proportion of cells in G₁ when cultured in low-Cl⁻ media compared with those cultured in 120 mM Cl⁻ (120 mM, 53.6 ± 2.0; 40 mM, 61.2 ± 4.7; 12 mM, 63.6 ± 2.0; % of cells in G₁, mean ± SE, n = 3, P < 0.05, repeated-measures ANOVA with Newman–Keuls multiple-comparisons posttest, Fig. 6A). There was also a significant decrease in the proportion of cells in S-phase (120 mM, 36.1 ± 1.0; 40 mM, 27.6 ± 4.3; 12 mM, 23.4 ± 3.3; % of cells in S-phase, means ± SE, n = 3, P < 0.05, repeated-measures ANOVA with Newman–Keuls multiple-comparisons posttest, Fig. 6B).
DISCUSSION

In this study, we show a new function for the recently discovered Ca^{2+}-activated Cl^- ion channel Ano1 as a regulator of cell proliferation. The contribution of Ano1 to normal CI^- transport (22) and a link to regulation of gastrointestinal motility has been previously demonstrated (12). However, a role for Ano1 as a regulator of proliferation has not been reported although it has been proposed, based on the expression of Ano1 expression in tumors (6). Changes in CI^- concentration have been associated with specific events of the cell cycle (2), but only a few CI^- channels have been directly linked to proliferation, specifically the CLIC3 and CLIC5 channels. Ano1 joins this group of CI^- channels.

We used multiple approaches to demonstrate a role of Ano1 in the regulation of proliferation. As markers for proliferation we used both Ki67 and EdU incorporation. Ki67 is a nuclear protein that is expressed in all stages of the cell cycle, except for the resting phase, G0. Ki67 has a very short half-life (60–90 min, Ref. 3), making its expression an accurate measure of proliferating cells. EdU is a 5-bromo-2-deoxyuridine (BrdU) analog that can easily be identified by immunohistochemistry or flow cytometry and is only incorporated into the cellular DNA during S-phase. Both methods gave similar results with a decrease in proliferation when Ano1 was genetically or pharmacologically inhibited, suggesting that indeed Ano1 regulates proliferation.

The experiments using whole mounts and primary cultures of ICC from Ano1^{+/+} mice showed that Ano1^{+/+} mice have fewer proliferating ICC. Furthermore, the experiments quantifying proliferating cells in the circular muscle layers of whole mounts from the mutant mice showed that the difference in proliferation in ICC was not detected in Kit-negative cells in a region; the circular muscle layer that we have shown does not contain Ano1-positive cells (8). To determine whether the actions of Ano1 were through its function as a Cl^- channel vs. another role for Ano1, we pharmacologically inhibited the channel and also carried out experiments depleting Cl^- in the media. Both sets of experiments yielded similar results, with a decrease in proliferation of ICC and CFPAC-1 cells when Cl^- entry was decreased. These results suggest that regulation of proliferation by Ano1 is related to its function as a Cl^- entry pathway.

The experiments with CI^- channel blockers are complicated by the lack of specificity of the broad-spectrum blockers used. We chose three structurally different blockers that had previously been shown to block Ano1 currents (11), but each of these are also well known to have other effects, including inhibition of other Cl^- channels (DIDS, Ref. 28), modulation...
of estrogen receptor signaling (tamoxifen, Ref. 14), and effects on K+ channels (7) and intracellular Ca2+ for niflumic acid (19). We therefore carried out the same experiments on ICC from Ano1(-/-) mice and showed that the block in proliferation by the channel blockers was abolished in cells from Ano1(-/-) mice. These data therefore support the argument that the decrease in proliferation induced by the nonspecific Cl- channel blockers was a specific effect on Ano1.

Proliferation was decreased but not abolished in Ano1(-/-) mice. This was an expected finding because multiple factors are known to regulate ICC proliferation including Kit (15) and serotonin (31, 34, 35), and these factors would be expected to maintain their effects on proliferation in the Ano1(-/-) mice and may also compensate for loss of Ano1. Although PND 0 Ano1(-/-) mice had decreased ICC proliferation, ICC network volumes were not significantly different from controls. This likely reflects the fact that ICC networks are not fully formed at birth and continue to develop until PND 11 (25). The presence of other factors that regulate ICC proliferation as well as the fact that there is extensive pruning of the ICC networks after birth (25) may explain the lack of change in network volumes. It would be interesting to look at the networks at a later time point, specifically after PND 11, but nearly all Ano1(-/-) mice die within the first 24 h of birth (23), making this experiment very challenging to complete. Furthermore, Cl- is an important factor for apoptosis (16), so it is possible that, despite the decrease in proliferation, we still would not be able to detect a difference in ICC network volume because ICC networks in the Ano1(-/-) mice may not undergo normal pruning. In primary cultures the numbers of ICC per field were modestly lower in cultures from knockout mice compared with cultures from control animals but only by 20%, probably attributable to the fact that cell counts and proliferation were by necessity measured at the same time point after plating the cells.

Our findings on the effect of Ano1 on proliferation of ICC and CFPAC-1 cells raise the question of whether Ano1 has a similar role in regulating proliferation in GISTs. GISTs arise from ICC and or ICC precursors (29). Although GISTs are responsive to inhibitors of Kit and PDGF receptor, these drugs are not curative (1) in part because the presence of Kit-low ICC stem cells in the tumor, which also express Ano1 (1). Ano1 may be important in the progression of GISTs, and, once specific inhibitors for Ano1 are developed, the potential role for these drugs in decreasing GIST proliferation can be tested.

The exact mechanism by which Ano1 regulates cellular proliferation remains to be elucidated. Our work suggests that Cl- entry is key to its mechanism of action. Furthermore, Ano1(-/-/-) mice have less phosphorylated Rb, which suggests...

![Fig. 6. Cell-cycle analysis. A: proportion of CFPAC-1 cells in G1 was increased when cultured in low-Cl- media. B: increase in G1 cells corresponded to a decrease in the proportion of cells in S-phase (*P < 0.05, repeated-measures ANOVA with Newman-Keuls posttest, n = 3).](image)

![Fig. 7. Small intestinal smooth muscle from PND 0, Ano1(-/-) mice had less phosphorylated retinoblastoma protein (Rb). Top: immunoblotting of protein from small intestine of Ano1(+/-) and Ano1(-/-) mice showed a decrease in the ratio of phosphorylated (Serine 780) Rb in Ano1(-/-) mice compared with controls. Bottom: graphical representation of peak band intensity (*P < 0.05, Mann Whitney nonparametric test, n = 7).](image)
that the effects of Cl− entry through Ano1 function at the G1/S transition. It is not known but would be interesting to study whether other Cl− entry pathways have similar effects on normal cell-cycle advancement and cellular proliferation. However, by using CFPAC-1 cells, we excluded a role for the cystic fibrosis transport regulator (CFTR), a Cl− transporter, in proliferation because of the presence of a loss of function mutation in the CFTR gene in CFPAC-1 cells (26).

In conclusion, the newly discovered protein Ano1 appears to be a regulator of proliferation in ICC and CFPAC-1 cells. The effect is specifically at the G1/S transition in CFPAC-1 cells, suggesting a new role for Ano1 in the control of cellular proliferation and tumorigenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


