Channel properties of the splicing isoforms of the olfactory calcium-activated chloride channel Anoctamin 2
Samsudeen Ponissery Saidu, Aaron B. Stephan, Anna K. Talaga, Haiqing Zhao, and Johannes Reisert
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The amino acid sequence shown in Fig. 1 D incorrectly included four amino acids encoded by exon 14 of ANO2 that is not found in olfactory cDNA and was not present in the clones used in the paper. GenBank accession numbers KC164759, KC164761, KC164760, and KC164762 have been updated accordingly. This error has no effect on any result or conclusion in the paper.

The corrected Fig. 1 is shown below.
Anoctamin (ANO)2 (or TMEM16B) forms a cell membrane Ca\(^{2+}\)-activated Cl\(^{-}\) channel that is present in cilia of olfactory receptor neurons, vomeronasal microvilli, and photoreceptor synaptic terminals. Alternative splicing of Ano2 transcripts generates multiple variants with the olfactory variants skipping exon 14 and having alternative splicing of exon 4. In the present study, 5′ rapid amplification of cDNA ends analysis was conducted to characterize the 5′ end of olfactory Ano2 transcripts, which showed that the most abundant Ano2 transcripts in the olfactory epithelium contain a novel starting exon that encodes a translation initiation site, whereas transcripts of the publically available sequence variant, which has an alternative and longer 5′ end, were present in lower abundance. With two alternative starting exons and alternative splicing of exon 4, four olfactory ANO2 isoforms are thus possible. Patch-clamp experiments in transfected HEK293T cells expressing these isoforms showed that N-terminal sequences affect Ca\(^{2+}\) sensitivity and that the exon 4–encoded sequence is required to form functional channels. Coexpression of the two predominant isoforms, one with and one without the exon 4 sequence, as well as coexpression of the two rarer isoforms showed alterations in channel properties, indicating that different isoforms interact with each other. Furthermore, channel properties observed from the coexpression of the predominant isoforms better recapitulated the native channel properties, suggesting that the native channel may be composed of two or more splicing isoforms acting as subunits that together shape the channel properties.
ANO1 channels form homo-multimers (Fallah et al., 2011; Sheridan et al., 2011), it raises the question whether ANO1 and ANO2 may form hetero-multimers.

Alternative splicing can greatly increase the differential functionality of proteins. Ano1 has at least six exons that are alternatively spliced, of which two encode for segments in the cytosolic N terminus (Caputo et al., 2008; Ferrera et al., 2009; O’Driscoll et al., 2011). ANO1 isoforms with different N termini display varied Ca$^{2+}$ sensitivity (Ferrera et al., 2009). We previously found that the olfactory Ano2 transcripts lack exon 14 (formerly annotated as exon 13 in Stephan et al., 2009), which is present in retinal Ano2 transcripts. Also, in the olfactory epithelium, alternative splicing occurs at exon 4 (exon 3 in Stephan et al., 2009), generating Ano2 variants either with or without exon 4. The exon 4–containing transcripts are present in greater abundance than the exon 4–lacking transcripts (Stephan et al., 2009). Exon 4 encodes a 35–amino acid segment within the cytoplasmic N terminus. The functional significance of such alternative splicing is unknown.

Previously, comparison between biophysical properties of native olfactory Ca$^{2+}$-activated Cl$^{-}$ channels and heterologously expressed ANO2 channels revealed similar channel properties with respect to the sensitivity of the channel to Ca$^{2+}$, single-channel conductance, “run-down” of the current, halide permeability, and current inactivation at negative holding potentials (Stephan et al., 2009). One notable exception is that, at positive holding potentials, unlike the native channel, the heterologously expressed ANO2 channel does not inactivate (Reisert et al., 2003; Pifferi et al., 2006, 2009, 2012; Stephan et al., 2008). One notable exception is that, at positive holding potentials, unlike the native channel, the heterologously expressed ANO2 channel does not inactivate (Reisert et al., 2003; Pifferi et al., 2006, 2009, 2012; Stephan et al., 2008). However, for channel function in this heterologous system. We also coexpressed the two variants of ANO2 (Isomorf A or B), together with their respective isoforms lacking the exon 4 sequence (Isoforms A$_{4A}$ or B$_{4A}$), and found that channel properties resulting from the coexpression of B isoforms, but not A isoforms, recapitulated properties of the native channel better than that of the individual expression of ANO2 isoforms. In particular, the channel resulting from coexpression of Isoforms B and B$_{4A}$, like the native channel, showed inactivation at positive holding potentials. These results suggest that the splicing isoforms form hetero-multimers and that the native channel may be composed of two or more splicing isoforms.

**MATERIALS AND METHODS**

5’ rapid amplification of cDNA ends (RACE) Mice were handled and euthanized with methods approved by the Animal Care and Use Committees of The Johns Hopkins University. Total olfactory epithelial RNA was extracted from adult C57BL/6 mice using Trizol (Invitrogen). The RNA was reverse transcribed using a RETROscript kit (Invitrogen) and an Ano2–specific primer (AS15) 5’-CTTTGGAGGAGTGGCATCTCCTG-3’, which binds within Ano2 exon 5. The single-stranded cDNA was purified using a Qiaquick PCR Purification kit (Qiagen). A poly-dT tail was added to the 3 ’ end of the single-stranded cDNA (corresponding to the 5 ’ end of the RNA) by a terminal transferase (New England Biolabs, Inc.) reaction for 5 min at 37°C. The enzyme was inactivated by incubation at 75°C for 10 min. Second-strand cDNA synthesis was performed by PCR with the AS13 primer and a primer that binds to the poly-dT tail and adds an “adapter sequence,” 5’-GACCTCGAGCGATCGATTGGTTTTTTTTT-3’ (Scotto-Lavino et al., 2006). A second round of PCR amplification was performed using a primer that only includes the adapter sequence 5’-GACTCGAGCGATCGATTGGTTTTTTTTTTT-3’ and an Ano2-specific primer nested upstream of AS15 primer, (AS134) 5’-CTCCAAGTCCTTCTGAGCTC-3’. The resulting PCR product was divided in two. One half of the product was column-purified by a Qiaquick PCR Purification kit and sequenced using an Ano2-specific primer nested upstream of AS134 primer, (Primer R1) 5’-GCCGACGAGCAGATTTGTTTG-3’. The other half of the reaction was analyzed by agarose gel electrophoresis. The gel was sliced into six sections of varying molecular weights, and DNA was extracted from each of the bands using a Qiaquick Gel Purification kit (Qiagen). The extracted DNA was TOPO TA cloned into pCR2.1 (Invitrogen), and the individual clones were sequenced by a plasmid-specific primer (M13).

**Differentiation of Ano2 mRNA variants by RT-PCR** Single-stranded cDNA from the 5’ RACE analysis was used as a template for PCR analysis. The reverse primer used in each of the reactions was Primer R1 (see 5’ RACE above), and the forward primers were: F1, 5’-ATGACATTGTTGCCAGGACACCA-3’; F2, 5’-GGACAAATCGATGAAATCTCC-3’; F3, 5’-GAGGGAATTCGCCGTTCCGG-3’; F4, 5’-CCCTCTGTCCTCCGATCCTC-3’; F5, 5’-GAGGAGCGCCGACGATTT-3’. Each of the PCR products was

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excised from the gel, reamplified by PCR, and TOPO-TA cloned and sequenced to verify their identities. A clone containing DNA amplified by the F5/R1 primer pair was used as a positive control template to test the primer efficacy for primers F3, F4, and F5. Clone 2-4 (Table S1), which contains the novel exon 1b, was used as a positive control template to test the primer efficacy of primer F2. A mixture of both positive control plasmids was used as a template to test the efficacy of Primer F1. Band densitometry quantification was performed using the “Analyze Gels” tool in ImageJ/FIJI (Fig. S1 C).

ANO2 isoform expression constructs
Expression constructs were all derived from the pAdTrack-cloned ANO2 expression construct used previously (Stephan et al., 2009). This construct encodes EGFP from a separate CMV promoter for positive identification of transfected cells but does not contain the full-length N-terminal–encoding sequence of either known ANO2 isoform. Therefore, the complete N-terminal–encoding sequences beginning at the most 5′-in-frame ATG were individually cloned at the 5′ end of this parent clone. To clone an expression construct encoding Isoform B, exon 1b and part of exon 3 were PCR amplified from clone 2-4 (Table S1) using the forward primer 5′-AGTCAAGGTACCATGAGAAGCTG-3′, which contains a KpnI recognition sequence, and the reverse primer 5′-CCACGAGCATCAGGTGTG-3′, which binds downstream of a Sall recognition sequence. The PCR product and parent plasmid were cut with Kpn1 and Sall, and the PCR insert was cloned into the parent plasmid. A similar approach was taken to clone the expression construct encoding Isoform A, but using the forward primer 5′-AGTCAAGGTACCATGAGAAGCTG-3′ and the clone containing DNA amplified by the F5/R1 primer pair as a template.

To generate the clones encoding the two "Δ4" isoforms, exon 4 was deleted from the above two clones by inverse PCR using primers 5′-ATGTCAAGGTACCATGAGAAGCTG-3′ and 5′-TCACAGTTGCTGCAGCCTG-3′, followed by 5′ phosphorylation and blunt-end self-ligation.

Immunohistochemistry
To generate C-terminal GFP fusion constructs with ANO2 Isoform B and Isoform BΔ4, the clones used for electrophysiological measurements were modified. Intervening plasmid sequence between the Ano2 open reading frame and the GFP open reading frame was deleted from each of the corresponding clones by inverse PCR with primers 5′-GTGACAGAACAGGAGAAGCCTG-3′ and 5′-TAGCTTTGCTGCTGGACCC-3′, and the PCR product was 5′ phosphorylated and blunt-end self-ligated, and the sequences were confirmed by sequencing.

HEK293T cells were grown in a 6-well plate on coverslips that were coated with poly-lysine. HEK293T cells (at ~50% confluency) were transfected with 0.5 µg of ANO2 Isoform B::GFP-encoding plasmid or ANO2 Isoform BΔ4::GFP-encoding plasmid using Lipofectamine 2000 (Invitrogen). After 36 h, the cells were washed with 1× PBS, pH 7.4, and a biotin solution (1.5 mg/ml biotin in 1× PBS, 1 mM CaCl2, and 0.5 mM MgCl2) was added to the cells. The cells were incubated at 4°C overnight with primary antibody in blocking buffer. Primary antibody, anti-GFP, was used at a dilution of 1:500 (Invitrogen). After washing, the coverslips were incubated for 2 h at room temperature with a secondary antibody conjugated to Alexa Fluor 488 (Invitrogen) and streptavidin Alexa Fluor 546 conjugate (Invitrogen). Coverslips were mounted in Vectashield (Vector Laboratories) containing DAPI stain and imaged with a confocal microscope (LSM 510 META; Carl Zeiss).

Electron microscopy
HEK293T cells were grown in a 6-well plate and transfected (at ~30% confluency) with 0.5 µg of either ANO2 Isoform B::GFP-encoding plasmid, ANO2 Isoform BΔ4::GFP-encoding plasmid, TrkA::GFP (provided by the Kuruvilla laboratory, Johns Hopkins University, Baltimore, MD), or pEGFP-N1 plasmid using Lipofectamine 2000 (Invitrogen). After 48 h, cells fixed in a sodium cacodylate (100 mM), 0.1% (vol/vol) gluteraldehyde, and 4% (vol/vol) paraformaldehyde solution in 1× PBS and stored in 100 mM sodium cacodylate. Next, cells were washed briefly in 1× PBS and resuspended in 1% low gelling-temperature agarose. The agarose blocks were trimmed into 1-mm3 pieces, cryoprotected by infiltration with 2.3 M sucrose/30% polyvinyl pyrolidone (10,000 mol wt)/PBS, pH 7.4, for 2 h, and mounted onto cryo-pins and rapidly frozen in liquid nitrogen. Ultrathin cryosections were cut on an ultramicrotome (UCT; Leica) equipped with an FCS cryoattachment and collected onto formvar/carbon-coated copper grids. The grids were then washed through several drops of 1× PBS containing 2.5% FCS and 10 mM glycine, pH 7.4, and then blocked in 10% FCS for 30 min and incubated overnight in chicken anti-GFP diluted to 1:400 (Abcam). After washing, the grids were incubated for 2 h in donkey anti–chicken 12-nm Au (Jackson ImmunoResearch Laboratories, Inc.). The grids were washed through several drops of 1× PBS followed by several drops of ddH2O; floated on a 1-nl drop of neutral uranyl acetate, pH 7.4, for 10 min; quickly washed through five drops of ddH2O; and floated onto an aqueous solution containing 3.2% polyvinyl alcohol (10,000 mol wt), 0.2% methyl cellulose (400 centiposites), and 0.1% uranyl acetate. The grids were then embedded by removing excess solution using hardened filter paper (no. 50; Whatman) and examined in a transmission electron microscope (Tecnai 12 Twin; FEI) operating at 100 kV. Images were collected using a digital camera (Soft Imaging System Megaview III; Olympus), and figures were assembled in Photoshop (Adobe) using only linear adjustments in brightness and contrast.

To quantify distances of each gold particle from the cell membrane, XY coordinates were obtained in ImageJ/FIJI of each particle and of a freehand line tracing the cell membrane. The distances between each particle and the closest point to the cell membrane line were calculated.

Patch-clamp electrophysiology
HEK293T cells were transfected with individual ANO2 expression constructs along with an expression plasmid for the principal subunit of the olfactory CNG channel CNGA2, which serves as a control for patch integrity (Stephan et al., 2009). The transfections were performed using the Lipofectamine 2000 reagent (Invitrogen). For coexpression experiments, the different ANO2 splice variants were expressed from individual plasmids transfected at equal amounts. Inside-out patches were excised using borosilicate glass pipettes fabricated using a puller (P97; Sutter Instrument). The pipettes had an open tip resistance of ~3 MΩ. Transfected cells were identified by their GFP fluorescence. The patches were pulled after a gigaohm seal was established. If, after excision, the membrane patch formed a vesicle at the pipette tip, it was opened by contact with debris at the bottom of the recording chamber. The patches were exposed to Ca2+ and CAMP immediately (15 s) after patch excision, and the protocol was continued for 20 min during which the chloride current stabilized after rundown. Peak ANO2 and CNGA2 currents were obtained from the first trace (15 s). Dose–response currents were recorded after the initial rundown of the chloride current subsided by exposing the patch to increasing concentrations of Ca2+. For quantifying the dose response,
the currents from each patch were normalized to the maximal current and the normalized values were averaged across multiple patches and fit with Hill functions to yield the $K_{1/2}$ (µM) and Hill coefficient ($n$) values. I-V relationships were obtained by preexposing the patches to 67 µM Ca$^{2+}$ for 4 s to eliminate the possibility of rectification induced as a result of inactivation of the ANO2 channel. The preexposure was followed by two voltage ramps (at 100 mV/s) from −50 to +50 mV and back to −50 mV, from which the traces were averaged to yield the I-V relationship. The currents were recorded using a patch-clamp amplifier (PC501A; Warner) and digitized using a data acquisition unit (Micro1401) and acquisition software (Signal; Cambridge Electronic Design). The currents were low-pass filtered (eight-pole Bessel; Krohn-Hite Corp.) at DC-100 Hz and DC-2 kHz, and digitized and sampled at 5 kHz. Two-way ANOVA was performed for comparing the inactivation of the splice variants (factor A, two levels) using $I_{\text{max}}/I_{\text{th}}$ values at the four concentrations (Factor B, 4 levels). The post-hoc test used was Bonferroni (P < 0.05). The analysis was performed using Origin (V.8.5) software. Shifts in dose–response relations were statistically compared using the $K_{1/2}$ values and errors obtained from the relevant dose–response relations. For whole-cell recordings, the pipettes had an open tip resistance of 5–5 MΩ. The recordings were made by varying the holding potential between −100 and +100 mV in steps of 20 mV, followed by a final step to −100 mV. Signals were filtered at 5 kHz and sampled at 10 kHz.

Solutions
For excised patch-clamp experiments, the bath solution and pipette solution contained (mM): 140 NaCl, 10 HEDTA, and 10 HEPES. For dose–response experiments, the different solutions contained 0.25, 0.75, 2.4, 11, and 67 µM of free Ca$^{2+}$ (Reisert et al., 2003), and patches were exposed from 0 to 10 s. To estimate the patch current magnitudes for the ANO2 and the CNGA2 currents, patches were exposed to 67 µM Ca$^{2+}$ from 0 to 3 s and to 1 mM cAMP from 6 to 9 s. The bath solution was used for “zero” Ca$^{2+}$ exposure. The pH was adjusted to 7.2 with NMDG. A fast-step perfusion system (Warner) was used to rapidly expose the patch and to switch solutions.

For whole-cell experiments, the extracellular solution contained (mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, and 10 HEPES, with pH adjusted to 7.4 using NaOH. The pipette solution contained (mM): 140 chloride chloride, 10 HEDTA, 10 HEPES, 3,209 CaCl$_2$ (1.5 µM of free Ca$^{2+}$) (Patton et al., 2004), with pH adjusted to 7.2 using NMDG. The holding potentials were corrected offline for liquid junction potential (−2.5 mV) calculated using Clampex software.

Online supplemental material
Fig. S1 A shows an agarose gel of the 5’ RACE PCR products for Ano2, whereas B and C show the reverse complement sequencing chromatogram of the predominant RACE PCR products including the new exon, named exon 1b (red), and densitometry quantification of bands from Fig. 1 (B and C). Fig. S2 shows current–voltage relationships and their rectification for Isoforms A and B at three different Ca$^{2+}$ concentrations. Fig. S3 quantifies the distance of immunogold particles from the cell membrane for Isoform B, B34, the TrkA receptor, and GFP when expressed in HEK cells. Figs. S1–S3 and Table S1 are available at http://www.jgp.org/cgi/content/full/jgp.201210937/DC1.

RESULTS
Olfactory Ano2 transcripts are expressed predominantly from a novel transcription initiation site
Subsequent to our publication in 2009 (Stephan et al., 2009), the mouse Ano2 sequence entry in GenBank was updated from NM_153589.1 to NM_153589.2, which indicated a change in the N-terminal amino acid sequence. To determine the 5’ end of Ano2 transcripts in the olfactory epithelium, we conducted 5’ RACE analysis (Scotto-Lavino et al., 2006). The 5’ RACE PCR products were analyzed by agarose gel electrophoresis, which showed two predominant bands at 532 and 436 bp, as well as a background smearing of lower molecular weights (Fig. S1 A). These PCR products were directly sequenced (Fig. S1 B) and were also cloned and then sequenced (Table S1). Contrary to the publicly available mouse Ano2 sequences (e.g., GenBank accession no. NM_153589.2), we found that the predominant olfactory Ano2 transcripts did not contain sequences from the first two exons. Instead, all of the olfactory Ano2 transcripts detected by 5’ RACE contained a novel starting exon, 206 bp in the longest RACE products, which we named exon 1b (Figs. 1 A and S1, A and B, and Table S1). Exon 1b is then joined to exon 3 and subsequent exons (Fig. 1 A). Based on the location of the most 5’ in-frame ATG codon, we determined that Ano2 transcripts containing exon 1b encode a shorter N terminus, containing a 7-amino acid sequence unique to this novel ANO2 isoform (Fig. 1 D).

Because this novel Ano2 exon 1b–containing variant has never been described, we asked whether the canonical Ano2 variant containing exon 1a and exon 2 was detectable in the olfactory epithelium, and if so, what its abundance is relative to the novel Ano2 variant containing exon 1b. We performed RT-PCR on olfactory epithelium cDNA with various primers that differentiate between the two variants. PCR using primers that bind to exon 3, common to all mRNA variants, readily amplified the target sequence, as did the primers specific to the novel exon 1b (Fig. 1 B; primer binding sites indicated in Fig. 1 A). Conversely, PCR using primers specific to exon 1a or exon 2 only weakly amplified the target sequences (Figs. 1 B and S1 C). To control for the possibility that this weak amplification reflected poor priming efficiency, we performed PCR under the same conditions but this time using cloned templates in equal amounts as positive controls. In this case, PCRs using each of the primer pairs readily amplified the target sequences (Figs. 1 C and S1 C). Thus, mRNA species representing both Ano2 5’ variants are present within the olfactory epithelium, but the novel exon 1b–containing Ano2 variant predominates over the canonical Ano2 isoform containing exon 1a and exon 2. For the sake of brevity, the two mRNA variants—the canonical exon 1a–and exon 2–containing variant and the novel exon 1b–containing variant—are referred to as encoding ANO2 Isoforms A and B, respectively (Fig. 1 A).

Given the two N termini of ANO2, along with the two additional possible splicing variants either containing or lacking exon 4 (Stephan et al., 2009), up to four different ANO2 isoforms may exist within the olfactory epithelium.
Isoforms A or B showed robust Ca\(^{2+}\) dose-dependent channel activity: currents rapidly peaked (I\(_{\text{max}}\)) and then inactivated at negative but less so at positive holding potentials (see below) over the course of the 10-s stimulation period to a lower current (I\(_{10\text{s}}\)) (Fig. 2, A–D). The normalized I\(_{\text{max}}\) values for the two individual ANO2 isoforms were fitted with Hill functions to obtain K\(_{1/2}\) values, which reflect the sensitivity of the channel activation by Ca\(^{2+}\) (Fig. 2, E and F). Comparison of K\(_{1/2}\) values between Isoforms A and B shows that Isoform B displayed an increased sensitivity to Ca\(^{2+}\) at a holding potential of \(-40\) mV as compared with Isoform A (Table 1; P = 0.04). This sensitivity increase is also apparent as a leftward shift of the dose–response relationship shown in Fig. 2 E. No significant sensitivity difference between the two isoforms was seen at the +40-mV holding potential (Table 1; P > 0.05; Fig. 2 F).

Although currents from Isoforms A and B showed inactivation at \(-40\) mV (Fig. 2, A and C), inactivation...
The I-V relationship for Isoforms A and B were similar, with the current showing a slight inward rectification and a reversal potential close to 0 mV (Fig. 2 H) at saturating (67 µM) Ca²⁺ concentrations. At intermediate Ca²⁺ concentration, both Isoform A and B channels displayed linear I-V relationships, and at low Ca²⁺ concentrations, both channels became outwardly rectifying (Fig. S2).

was not apparent at +40 mV, particularly at high Ca²⁺ concentrations (Fig. 2, B and D). To quantify inactivation, the ratios of $I_{\text{max}} / I_{10s}$ were calculated (Fig. 2 G). The $I_{\text{max}} / I_{10s}$ ratios approach unity at +40 mV for both Isoforms A and B at high Ca²⁺ concentrations, indicating a lack of inactivation under these conditions (Fig. 2 G). It has been shown that native olfactory Ca²⁺-activated Cl⁻ channels exhibit inactivation even at +40 mV and at high Ca²⁺ concentrations (Reisert et al., 2003); thus, neither Isoform A nor Isoform B in this heterologous system fully recapitulates the native olfactory channel properties.

The I-V relationship for Isoforms A and B were similar, with the current showing a slight inward rectification and a reversal potential close to 0 mV (Fig. 2 H) at saturating (67 µM) Ca²⁺ concentrations. At intermediate Ca²⁺ concentration, both Isoform A and B channels displayed linear I-V relationships, and at low Ca²⁺ concentrations, both channels became outwardly rectifying (Fig. S2).

Table 1: Summary of Ca²⁺ sensitivities of the ANO2 isoforms

<table>
<thead>
<tr>
<th>Values derived from $I_{\text{max}}$</th>
<th>Isoform A</th>
<th>Isoforms A + Aₐ₀</th>
<th>Isoform B</th>
<th>Isoforms B + Bₐ₀</th>
<th>Native channel (mouse)</th>
<th>ANO2 variant</th>
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<tbody>
<tr>
<td>$K_{1/2}$ (µM)</td>
<td>2.1 ± 0.26</td>
<td>1.65 ± 0.13</td>
<td>1.33 ± 0.13</td>
<td>2.04 ± 0.20</td>
<td>3.46 ± 0.21</td>
<td>1.83 ± 0.03</td>
</tr>
<tr>
<td>n Hill Coeff.</td>
<td>1.79 ± 0.38</td>
<td>1.91 ± 0.23</td>
<td>2.49 ± 0.39</td>
<td>2.61 ± 0.81</td>
<td>1.41 ± 0.1</td>
<td>2.3 ± 0.07</td>
</tr>
<tr>
<td>$I_{10s}/I_{\text{max}}$ at 67 µM</td>
<td>0.62 ± 0.06</td>
<td>0.4 ± 0.05</td>
<td>0.55 ± 0.07</td>
<td>0.43 ± 0.07</td>
<td>0.59 ± 0.05</td>
<td>0.41 ± 0.06</td>
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All values are mean ± SEM (6–11 patches). Mouse-native channel values are from Reisert et al., 2005, and Table S2 in Stephan et al., 2009.
Figure 3. Patch-clamp analysis of ANO2 isoforms containing or lacking exon 4-encoded sequence. (A) 
Ca²⁺ and cAMP-gated currents from HEK293T patches expressing the individual isoforms of ANO2 along with the CNGA2 channel. Patches were exposed immediately (15 s) after excision, and the holding potential was −40 mV. (B) Quantification of the peak ANO2 and CNGA2 currents for each expression condition derived from experiments as shown in A. Current (pA) values are means from 9–11 patches ± SEM. *, P < 0.01; **, P < 0.001; Student’s t test.

Exon 4 is required to encode functional ANO2 channels
To determine if the exon 4–encoded protein sequence confers any functional differences to the heterologously expressed ANO2 channels, we expressed Isoforms A, B, A4, and B4 individually in HEK293T cells and performed excised inside-out patch-clamp analysis. Whereas Isoforms A and B produced robust chloride currents in response to a saturating Ca²⁺ concentration (67 µM), Isoforms A4 and B4 yielded negligible current amplitudes (Fig. 3 A). The traces in Fig. 3 A represent results from an experimental protocol where membrane patches from cells cotransfected with plasmids encoding each of the four isoforms of ANO2 along with a plasmid encoding the principal subunit of the olfactory CNG channel CNGA2 were exposed to 67 µM Ca²⁺ for 3 s followed by 1 mM cAMP for 3 s. The traces were quickly recorded 15 s after patch excision to minimize the effect of Cl⁻ current rundown. The cAMP-induced currents through CNGA2 served as controls for patch integrity. Although cells expressing the “Δ4” isoforms did not show any chloride currents, cAMP-generated currents were still observed. A quantification of the amplitude of the ANO2 and CNGA2 currents is shown in Fig. 3 B. Although Isoforms A and B exhibited considerable chloride current amplitudes (160 ± 34 and 105 ± 40 pA, respectively), Isoforms A4 and B4 yielded negligible current amplitudes (−1.2 ± 0.7 and −0.3 ± 0.7 pA, respectively). The cAMP currents for all four isoforms remained, on average, the same (159 ± 66, 171 ± 63, 166 ± 91, and 170 ± 47 pA).

To determine whether the absence of currents from ANO2 “Δ4” isoforms is caused by “instantaneous rundown” after patch excision, we performed experiments in the whole-cell configuration where rundown is not observed (Pifferi et al., 2009). We transfected HEK293T cells with Isoform B, which we determined to be the predominant isoform in the olfactory epithelium, and Isoform B4 individually and used a pipette solution containing 1.5 µM Ca²⁺ to activate whole-cell currents (Fig. 4, A and B). The cells expressing Isoform B showed robust outwardly rectifying currents (Fig. 4 C), which is a known property of ANO2 at subsaturating Ca²⁺ levels (Pifferi et al., 2009). In cells expressing Isoform B4, the currents were similar in magnitude to those in untransfected cells (Fig. 4 C). Collectively, the results from excised patch recordings and whole-cell recordings indicate
that the protein sequence encoded by Ano2 exon 4 is necessary for channel function or membrane targeting in this heterologous system.

Cellular localization of alternatively spliced isoforms

To address if exon 4–lacking isoforms traffic to the cell membrane and thus if exon 4 is required for cell membrane targeting, we investigated the protein localization of GFP-tagged ANO2 isoforms expressed in HEK cells. First, we expressed either Isoform B::GFP or BΔ4::GFP and visualized the fusion proteins with anti-GFP immunofluorescence staining. The cell membrane of HEK293T cells was labeled with biotin and stained with a streptavidin Alexa Fluor 546 conjugate (Fig. 5 A). Biotin labeling (red) was clearly limited to the cell membrane where it colocalized with Isoform B::GFP staining (green), which was more broadly distributed throughout the cell (Fig. 5 A, top row, arrowhead). A similar membrane colocalization of the GFP and biotin staining was also observed for the B isoform lacking exon 4 (Fig. 5 A, bottom row, arrowhead).

In a second approach, we investigated protein localization at a higher resolution using electron microscopy and immunogold labeling. We expressed Isoform B::GFP and BΔ4::GFP, as well as TrkA::GFP and GFP as controls. TrkA, the nerve growth factor receptor, is a well-characterized membrane protein. As shown in Fig. 5 B (bottom left), TrkA::GFP immunogold particles are clearly localized to the cell membrane, whereas GFP particles are distributed throughout the cytoplasm (bottom right). Immunogold particles for Isoform B::GFP (Fig. 5 B, top left) and Isoform B lacking exon 4 (Fig. 5 B, top right) are, similar to TrkA::GFP particles, found predominately close to the cell membrane. We measured the distances of gold particles to the cell membrane for all four proteins (Fig. S3). The distance distributions for B::GFP and BΔ4::GFP, which are similar to TrkA::GFP distribution, suggest a membrane localization. These data show that BΔ4::GFP is indeed capable of targeting to the cell membrane and that the exon 4 sequence is necessary for channel function.

Rundown properties of individual and coexpressed splice variants

Given that ANO2 isoforms lacking exon 4 cannot confer ANO2 channel activity to HEK293T cells when expressed individually, we considered the possibility that the “Δ4” isoforms could constitute modulatory subunits of a hetero-multimeric ANO2 channel. To test this possibility, we cotransformed HEK293T cells with expression constructs encoding both Isoforms A and AΔ4 and alternatively also B and BΔ4, performed excised inside-out patch-clamp analysis, and asked whether coexpression affected channel properties. Some of the analyses were performed as described above for individual expression of isoforms; thus, comparisons were made directly to the channel properties of Isoforms A or B, respectively, when expressed alone.

We first investigated the rundown properties of channels arising from expression of a single isoform or when coexpressed with the corresponding isoform lacking exon 4. Patches were repeatedly exposed to 67 µM Ca²⁺ and 1 mM cAMP using the same protocol as shown in Fig. 3 A. The CNG current was also recorded to monitor patch integrity over time. When Isoform A was expressed alone, the peak Cl⁻ current declined quickly with each repeated Ca²⁺ exposure (Fig. 6 A) and lost nearly half its current within 1 min (Fig. 6 E). In contrast, coexpression
of A with A_{4A} greatly slowed the rundown of the Cl⁻ current (Fig. 6, C and E). Expression of Isoform B alone or together with B_{34} yielded channels that had similar rundown rates falling in between those of A and A + A_{34} (Fig. 6, B, D, and E). We tested for statistical difference between the different expression conditions at 1 min after patch excision, where the difference in rundown was largest. The level of rundown was different between A and A + A_{34} (P = 0.024) but not between other combinations. After 10 min after excision, all four channels decreased to similar current levels. The CNG current remained quite stable over time, as indicated by the closed symbols at t = 10 min in Fig. 6 E.

Coexpression of Isoforms A and A_{34} results in altered channel sensitivity

We exposed patches that coexpressed Isoforms A and A_{34} to the same set of Ca²⁺ concentrations used in Fig. 2 for 10 s and determined the Ca²⁺ sensitivity and inactivation properties of the resulting channel at a holding potential of −40 and +40 mV (Fig. 7, A and B). At −40 mV, the channel resulting from coexpression of Isoforms A and A_{34} had similar sensitivities (Table 1; K_{1/2} = 1.65 ± 0.13 µM) compared with Isoform A alone (Fig. 7 D), whereas at +40 mV, coexpression yielded a more sensitive channel (Fig. 7 E; K_{1/2} = 0.96 ± 0.09 µM; P = 0.017). However, the coexpression increased the inactivation of the current during the 10-s Ca²⁺ exposure at −40 mV (P < 0.01), a property that remained unaltered at +40 mV (Fig. 7 C). The inactivation was again quantified by dividing the current at 10 s (end of stimulation, I_{10s}) by the maximal current (I_{max}) and was plotted as a function of Ca²⁺ concentration. Little difference was seen between the I-V relationship of Isoform A and Isoform A coexpressed with Isoform A_{34} (Fig. 7 F).

Coexpression of Isoforms B and B_{34} results in altered channel inactivation properties

We found that when Isoforms B and B_{34} were coexpressed (Fig. 8, A and B), ANO2 channel activity was

**Figure 6.** Rundown of the Cl⁻ current upon coexpression of ANO2 isoforms. Expression of Isoforms A and B alone and with their respective isoforms lacking exon 4 yielded channels with different rundown properties. (A) Isoform A. (B) Isoform B. (C) Isoform A + A_{34}. (D) Isoform B + B_{34}. (E) Rundown as a function of time after patch excision. All currents were normalized to the first current recorded 15 s after excision. *, P < 0.05 for Isoform A compared with A + A_{34} at t = 1 min. Number of patches recorded from for Isoform A, B, A + A_{34}, and B + B_{34} were 7, 5, 12, and 7, respectively. The holding potential was −40 mV.
less sensitive to Ca\(^{2+}\) than when Isoform B was expressed alone, which is opposite to what was observed for Isoforms A and A\(_{44}\) at positive holding potentials. This decreased sensitivity was apparent as a rightward shift of the Ca\(^{2+}\) dose–response curve (Fig. 8, D and E) and as an increase in \(K_{1/2}\) values (Table 1; \(P = 0.01\)). The \(K_{1/2}\) value for the coexpression condition (2.04 ± 0.2 µM) is closer to that of the native channel (3.46 ± 0.21 µM) at a holding potential of −40 mV. At a holding potential of +40 mV, the \(K_{1/2}\) value for the coexpression (1.45 ± 0.19 µM) is comparable to that of the native channel (1.48 ± 0.08 µM; Table 1) (Stephan et al., 2009).

We also found that coexpression changed channel inactivation properties to more closely resemble the native olfactory channel. At a holding potential of +40 mV, whereas the individually expressed Isoforms A or B, or the coexpressed Isoforms A and A\(_{44}\), did not show inactivation at higher concentrations of Ca\(^{2+}\) (Figs. 2, B, D, and G, and 7, B and C), coexpression of Isoforms B and B\(_{44}\) showed remarkable inactivation (Fig. 8, B and C). A significant difference between Isoform B and the coexpression is observed in the inactivation properties at both positive and negative holding potentials (\(P < 0.05\)). This display of inactivation even at positive holding potentials is reminiscent of what is observed for the native olfactory channel (Stephan et al., 2009) (Table 1). However, a complete recapitulation in the degree of inactivation at high Ca\(^{2+}\) levels seen in the native channel is not observed in the coexpression (Table 1). The I-V relationship of Isoform B is not different from the coexpression; both Isoform B alone and Isoform B coexpressed with Isoform B\(_{44}\) showed a slight inward rectification and a reversal potential close to 0 mV (Fig. 8 F).

**DISCUSSION**

In this study, we describe the identification of a novel exon encoding a novel N terminus of the olfactory

![Figure 7](image)

Figure 7. Channel properties of Isoform A coexpressed with Isoform A\(_{44}\). (A and B) Currents from the coexpression of ANO2 Isoforms A and A\(_{44}\) at holding potentials of −40 and +40 mV in response to varying concentrations of Ca\(^{2+}\). (C) Comparison of current inactivation of the coexpression with that of Isoform A alone across all Ca\(^{2+}\) concentrations (data for A replotted from Fig. 2 G for comparison). *, \(P < 0.01\); two-way ANOVA. (D and E) Hill fits of normalized current values from the coexpression of Isoforms A and A\(_{44}\) at holding potentials of −40 and +40 mV (eight and six patches, respectively). Hill fits of currents from Isoform A (from Fig. 2, E and F) are also drawn for comparison. (F) I-V relationship at saturating Ca\(^{2+}\) for the coexpression and its comparison with Isoform A.

![Figure 8](image)

Figure 8. Channel properties of Isoform B coexpressed with Isoform B\(_{44}\). (A and B) Currents from the coexpression of ANO2 Isoforms B and B\(_{44}\) at holding potentials of −40 and +40 mV in response to varying concentrations of Ca\(^{2+}\). (C) Comparison of current inactivation of the coexpression (seven patches) with that of Isoform B alone across all Ca\(^{2+}\) concentrations (data for Isoform B replotted from Fig. 2 G for comparison). *, \(P < 0.01\); **, \(P < 0.0001\); two-way ANOVA. (D and E) Hill fits of normalized current values from the coexpression of Isoforms B and B\(_{44}\) at holding potentials of −40 and +40 mV (seven patches). Hill fits of currents from Isoform B (from Fig. 2, E and F) are also drawn for comparison. (F) I-V relationship at saturating Ca\(^{2+}\) for the coexpression and its comparison with Isoform B.
Ca\textsuperscript{2+}-activated chloride channel ANO2. The ANO2 isoform containing this novel N terminus along with the exon 4 sequence (Isoform B) is the predominant olfactory isoform and has current properties very similar to the previously reported isoform with the truncated N terminus starting at exon 3 (Stephan et al., 2009). Probably most importantly for this study, the inactivation properties at positive and negative holding potentials were similar between these two isoforms. We also found the presence of a rarer isoform (Isoform A) that conforms to the longer N terminus encoded by the publically available sequence (GenBank accession no. NM_153589.2). When activated by Ca\textsuperscript{2+}, Isoform A produced similarly sized chloride currents and had similar current inactivation kinetics at +40 and −40 mV compared with Isoform B. However, Isoform A was less sensitive to Ca\textsuperscript{2+} at −40 mV, indicating that the N terminus is involved in Ca\textsuperscript{2+} sensitivity at negative potentials. A similar scenario has been reported for ANO1, where an alternatively spliced protein segment (22 amino acids) in the cytosolic N terminal results in a fourfold difference in Ca\textsuperscript{2+} sensitivity among the isoforms, with the shorter isoform being more sensitive than the longer isoform, especially at negative holding potentials (Ferrera et al., 2009). Although the difference in K\textsubscript{l/2} values in the ANO2 counterparts are not as drastically different as in the ANO1 isoforms, the results nonetheless point to the importance of the N terminus in determining the Ca\textsuperscript{2+} sensitivity of these channels (Table 1).

We also exposed patches expressing two additional isoforms that splice alternatively at exon 4 and contain the alternative N termini (exon 1b or exon 1a) to Ca\textsuperscript{2+} (Isoform A\textsubscript{34} and Isoform B\textsubscript{34}). Regardless of the starting exon, the isoforms lacking the exon 4 sequence failed to generate any current when exposed to Ca\textsuperscript{2+}, indicating that without the exon 4 sequence, these isoforms either do not target to the cell membrane or the channels are nonfunctional. Immunohistochemical and immunogold data (Fig. 5) show that isoforms lacking exon 4 can traffic to the cell membrane, suggesting that exon 4 is integral to channel function and not required for membrane trafficking. The ANO channel pore is predicted to be between transmembrane domains 5 and 6 (Caputo et al., 2008; Das et al., 2008; Yang et al., 2008), thus excluding the exon 4 sequence, which is predicted to be within the N-terminal cytoplasmic domain. Therefore, it is possible that exon 4 encodes a regulatory domain that is crucial for channel function. A web-based calmodulin (CaM)-binding prediction application shows Ano2exon 4 partially coding for a putative CaM-binding domain, which may act as a regulator of channel activity (Calmodulin Target Database; Ikura laboratory, University of Toronto, Toronto, Canada) (Fig. 1 D). In the case of ANO1, it has been demonstrated that CaM physically interacts with homologous N-terminal putative CaM-binding sites and that CaM is necessary for channel activation (Tian et al., 2011). However, the splicing variants of Ano1 not encoding the CaM-binding domain were still able to elicit currents and did not depend on CaM for activation. In the absence of any evidence to the contrary, CaM-dependent activation of ANO2 cannot be completely ruled out, although the native channel does not seem to be affected by exogenously applied CaM (Reisert et al., 2003).

As exon 4–lacking isoforms do not form functional channels on their own, we investigated if these isoforms could modulate channel properties of the exon 4–containing isoforms when expressed together. Indeed, coexpression of Isoform A with A\textsubscript{34} yielded a channel that displayed significantly slowed rundown properties compared with those when A was expressed alone. The latter displayed very fast rundown kinetics, losing nearly half its current within 1 min after patch excision (Fig. 6). Such a difference was not observed when Isoform B and B\textsubscript{34} were coexpressed, indicating that the speed of rundown might be determined by the interplay of the exon 4 sequence of Isoform A with its longer N terminus.

A second biophysical property altered by coexpression of isoforms containing and lacking exon 4 is the inactivation property of the channel during longer Ca\textsuperscript{2+} exposures. Interestingly, when Isoform A was coexpressed with A\textsubscript{34}, the channel showed increased inactivation at −40 mV but did not alter its inactivation properties at +40 mV. This is in contrast to the channel resulting from coexpression of Isoform B and B\textsubscript{34}, where the channel inactivated more at both holding voltages and became less sensitive to Ca\textsuperscript{2+} (Figs. 7 and 8). Current properties of the coexpression resemble the native olfactory Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel more closely (see Table 1). In particular, the currents of the Isoform B coexpression show a slow inactivation even at positive holding potentials, similar to what is observed for the native channel. The peak current value (198 ± 50 pA) of the coexpressed B isoforms is not significantly different from the expression of Isoform B alone (105 ± 40 pA; P > 0.05), indicating that coexpression does not alter membrane trafficking of the predominant isoform. It is tempting to speculate that the native olfactory Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel might be a heteromer composed of (at least) two ANO2 isoforms, predominantly the B + B\textsubscript{34} isoforms, as coexpression of A isoforms or expression of Isoform B alone does not recapitulate the native channel. It has been shown that ANO1 strictly forms homodimers by associating before reaching the cell membrane (Fallah et al., 2011; Sheridan et al., 2011) and that ANO1 and ANO2 are both present in microvilli of vomeronasal neurons (Dibattista et al., 2012). It has also been shown that different ANOs can interact and alter the activities of each other. For example, ANO9 can negatively affect the activity of ANO1 when expressed in FRT cells (Schreiber et al., 2010). Therefore, it is also possible that ANO2 B isoforms, instead of forming heteromers,
may be interacting in other ways to modify their current properties.

To summarize, we showed that the major olfactory isoform of ANO2 is composed of a novel N terminus that contributes toward the Ca\textsuperscript{2+} sensitivity of the channel. The previously reported splice site exon 4 sequence is indispensable for channel function through a yet unidentified mechanism. Coexpression of the predominant isoform of ANO2 is indispensable for channel function through a yet unidentified mechanism.

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Note added in proof. In a recent paper by Tien et al. (2013. Proc. Natl. Acad. Sci. USA.http://dx.doi.org/10.1073/pnas.1303672110), the authors describe a 19-amino acid domain in the N terminus of Anoctamin 1 that can support dimerization. Interestingly, the region homologous to this domain in Anoctamin 2 is entirely contained within exons 4, the exon that can be spliced out in olfactory receptor neurons.

REFERENCES


Olfactory Anoctamin 2 isoforms


