ANO1 in intramuscular interstitial cells of Cajal plays a key role in the generation of slow waves and tone in the internal anal sphincter

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Key points

- The internal anal sphincter develops tone important for maintaining high anal pressure and continence. Controversy exists regarding the mechanisms underlying tone development.
- We examined the hypothesis that tone depends upon generation of electrical slow waves (SWs) initiated in intramuscular interstitial cells of Cajal (ICC-IM) by activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (ANO1, encoded by Ano1) and voltage-dependent L-type Ca\(^{2+}\) channels (Cav\(_{L}\), encoded by Cacna1c).
- Measurement of membrane potential and contraction indicated that ANO1 and Cav\(_{L}\) have a central role in SW generation, phasic contractions and tone, independent of stretch.
- ANO1 expression was examined in wildtype and Ano1\(^{+/egfp}\) mice with immunohistochemical techniques. Ano1 and Cacna1c expression levels were examined by quantitative PCR in fluorescence-activated cell sorting.
- ICC-IM were the predominant cell type expressing ANO1 and the most likely candidate for SW generation. SWs in ICC-IM are proposed to conduct to smooth muscle where Ca\(^{2+}\) entry via Cav\(_{L}\) results in phasic activity that sums to produce tone.

Abstract The mechanism underlying tone generation in the internal anal sphincter (IAS) is controversial. We examined the hypothesis that tone depends upon generation of electrical slow waves (SWs) initiated in intramuscular interstitial cells of Cajal (ICC-IM) by activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (encoded by Ano1) and voltage-dependent L-type Ca\(^{2+}\) channels (encoded by Cacna1c). Phasic contractions and tone in the IAS were nearly abolished by ANO1 and Cav\(_{L}\) antagonists. ANO1 antagonists also abolished SWs as well as transient depolarizations that persisted after addition of Cav\(_{L}\) antagonists. Tone development in the IAS did not require stretch of muscles, and the sensitivity of contraction to ANO1 antagonists was the same in stretched versus un-stretched muscles. ANO1 expression was examined in wildtype and Ano1\(^{+/egfp}\) mice with immunohistochemical techniques. Dual labelling revealed that ANO1 expression could be resolved in ICC but not smooth muscle cells (SMCs) in the IAS and rectum. Ano1, Cacna1c and Kit gene expression were the same in extracts of IAS and rectum muscles. In IAS cells isolated with fluorescence-activated cell sorting, Ano1 expression was 26.5-fold greater in ICC than in SMCs while Cacna1c expression was only 2-fold greater in SMCs than in ICC. These data support a central role for ANO1 and Cav\(_{L}\) in the generation of SWs and tone in the IAS. ICC-IM are the probable cellular candidate for ANO1 currents and SW generation. We propose that ANO1 and Cav\(_{L}\) collaborate to generate SWs in ICC-IM followed by conduction to adjacent SMCs where phasic calcium entry through Cav\(_{L}\) sums to produce tone.

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**Introduction**

In spite of the importance of the internal anal sphincter (IAS) in maintaining faecal continence, controversy remains regarding the mechanisms underlying tone generation. Some investigators have suggested that the IAS is a ‘purely tonic muscle’ (Patel & Rattan, 2006), with characteristics similar to tracheal smooth muscles or blood vessels such as the aorta, and that tone is largely the result of biochemical mechanisms. We have shown that the IAS of mice, canines and monkeys is a phasic muscle (Mutafova-Yambolieva et al. 2003; Cobine et al. 2007; McDonnell et al. 2008; Duffy et al. 2012) and concluded that tone develops as a consequence of incomplete relaxation between contractions. Thus, in our concept fusion of phasic contractions is essential for maintenance of a zone of high pressure within the anal canal. Dysregulation of IAS tone and pressure within the anal canal can contribute to faecal incontinence and other defecatory disorders.

In most regions of the gastrointestinal (GI) tract phasic contractile activity results from the generation and propagation of electrical slow waves (SWs). Interstitial cells of Cajal (ICC) are the pacemaker cells that generate SWs (Sanders et al. 2014). SWs also occur spontaneously (i.e. in the absence of exogenous stimuli) in the IAS and a population of intramuscular ICC (ICC-IM) exists in these muscles (Horiguchi et al. 2003; Cobine et al. 2010; Hall et al. 2014). However, the cell that generates SWs in the IAS remains uncertain. Based on studies in other GI regions and our own work on the IAS we propose that excitation–contraction coupling in the IAS includes: (i) generation of SWs by ICC-IM; (ii) conduction of SWs to electrically coupled smooth muscle cells (SMCs); (iii) depolarization and activation of voltage-dependent L-type Ca\(^{2+}\) channels (Cav\(_L\)); (iv) Ca\(^{2+}\) entry; and (v) initiation of contraction. In most regions of the gut SW frequency is such that complete relaxation is accomplished during the period between SWs. This, however, is not the case in the IAS where SW frequencies averaging 70 cycles min\(^{-1}\) (c.p.m.) were observed at the distal end of the mouse IAS (Hall et al. 2014).

A significant part of SW depolarization appears to be due to openings of Ca\(^{2+}\)-activated Cl\(^-\) channels, encoded in ICC by Ano1. Previous studies have shown that: (i) Ano1 (formerly Tmem16a) transcripts are highly expressed in ICC of the small intestine (Chen et al. 2007); (ii) Ano1 protein can only be resolved by immunohistochemistry in ICC within GI muscles (Hwang et al. 2009; Gomez-Pinilla et al. 2009); (iii) ICC express Ca\(^{2+}\)-activated Cl\(^-\) currents consistent with the properties of Ano1 currents expressed in model systems (Zhu et al. 2009); (iv) SWs fail to develop in Ano1 null mice (Hwang et al. 2009); and (v) SWs and phasic contractions are greatly diminished or abolished by blockers of ANO1 channels in the stomach and intestine (Hwang et al. 2016; Sanders et al. 2012; Singh et al. 2014).

In contrast to these findings a recent study of IAS muscles reported that tone involves a unique population of SMCs in the IAS that express ANO1 (Zhang et al. 2016). Since spontaneous activation of ANO1 was not observed in isolated SMCs it was suggested that muscle stretch may lead to activation of ANO1 by providing a global rise in intracellular calcium. It is unclear how such a mechanism might correspond to or contribute to the phasic electrical and mechanical behaviours of IAS muscles. If rationales for therapies are to be developed for treating IAS dysfunction, it will be necessary to obtain an accurate concept of the physiology of IAS motor function.

The present study was designed to address the following questions: (i) Is stretch required for development of tone in the IAS? (ii) To what extent are electrical SWs, phasic contractions and tone modulated by blockers of ANO1 and Cav\(_L\)? (iii) Are there differences in the gene and protein expression patterns of ANO1 and Cav\(_L\) in the IAS versus the rectum (which does not generate tone)? (iv) What cell type(s) within the muscularis externa of the IAS express ANO1 and Cav\(_L\)? Our results support an important role for ANO1 and Cav\(_L\) in the IAS, but the cells and means by which these channels contribute to the development of tone differ substantially from that previously reported by Zhang et al. (2016).

**Methods**

**Ethical approval**

Experiments and procedures performed in this study were approved by the Institutional Animal Use and Care Committee at the University of Nevada, Reno. All mice used were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
Animals
Mice of either sex (1.5–4 months of age) were killed with isoflurane (Baxter, Deerfield, IL, USA) and then cervically dislocated. C57BL/6 (wild-type, WT; n = 95), Kit<sup>cre-GFP/+</sup> (n = 24), Pdgfra<sup>cre-GFP/+</sup> (n = 15) and smMHC<sup>Cre-egfp</sup> (n = 21) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Ano1<sup>tm1Jrr</sup> mice (referred to in this paper as Ano1<sup>tm1Jrr</sup>, n = 6) were received as a gift from Dr Jason Rock, University of California, San Francisco, and were generated as previously described (Huang et al. 2012).

Tissue preparation
Rectoanal muscles were pinned in a dissection dish and dissected in ice cold Krebs–Ringer bicarbonate solution (KRBS) of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 23.8 NaHCO<sub>3</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 11 dextrose. For cell-sorting experiments, tissues were dissected in cold Ca<sup>2+</sup>-free KRBS. KRBS had a pH of 7.4 after bubbling to equilibrium with 95% O<sub>2</sub>/5% CO<sub>2</sub>. IAS muscle strips consisted of the final 1 mm of the GI tract while a 1.5 mm wide strip of muscle located 2.5–4 mm from the distal extremity was used for experiments examining gene and protein expression in rectum.

Immunohistochemistry
Whole mount preparations. Whole rectoanal tissues (final 3.5 mm of the GI tract) were pinned in a Sylgard bottomed dish and fixed for 15 min with ice-cold acetone, washed in 0.1 M PBS for 4 h before blocking with 1% BSA for 1 h at 20°C to prevent non-specific binding. Tissues were subsequently incubated in the first primary antibody (mSCFR, anti-Kit antibody, R&D Systems, Minneapolis, MN, USA; 1:1000 dilution) with a 0.5% Triton-X working solution for 48 h at 4°C. Following incubation in the first primary antibody, tissues were washed in 0.1 M PBS for 4 h and subsequently incubated in secondary antibody (Alexa Fluor anti-goat 594 (Kit) or Alexa Fluor anti-rabbit 488, Invitrogen; 1:1000 dilution). Subsequently sections were washed and incubated in 1% BSA in preparation for incubation with the second primary antibody [i.e. mSCFR, anti-Kit antibody, R&D Systems; 1:1000 dilution or anti-smooth muscle myosin heavy chain (smMHC), Biomedical Technologies Inc., Stoughton, MA, USA; 1:100 dilution]. Following incubation with the second primary antibody, sections were washed and incubated with secondary antibody [Alexa Fluor anti-goat 594 (Kit) or Alexa Fluor anti-rabbit 594 (smMHC), Invitrogen; 1:1000 dilution] as described above. After washing with PBS, slides were covered with coverslips using Aquamount mounting medium (Lerner Laboratories, Pittsburgh, PA, USA).

Fluorescence-activated cell sorting
Cell dispersion. IAS muscle strips (1 mm) were obtained from Kit<sup>cre-GFP</sup>, Pdgfra<sup>cre-GFP</sup> and smMHC<sup>Cre-egfp</sup> mice as previously described (Cobine et al. 2014). Tissues were cut into four to five smaller pieces parallel to the circular muscle before being incubated in an enzymatic cocktail consisting of 4 mg ml<sup>−1</sup> collagenase type II (Worthington Biochemical, Lakewood, NJ, USA), 8 mg ml<sup>−1</sup> BSA (Sigma-Aldrich, St. Louis, MO, USA) and 8 mg ml<sup>−1</sup> trypsin inhibitor (Sigma-Aldrich) at 37°C for 30 min. To remove all remaining enzymes, tissues were then washed three times in Hanks’ solution consisting of (in mM): 125 NaCl, 5.36 KCl, 15.5 NaHCO<sub>3</sub>, 0.336 Na<sub>2</sub>HPO<sub>4</sub>, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 2.9 sucrose and 11 HEPES adjusted to a pH of 7.2 with NaOH. Tissues were then triturated (w/v) paraformaldehyde for 15 min at 20°C. Tissues were subsequently washed, dehydrated and frozen as previously described (Cobine et al. 2011; Hall et al. 2014). Frozen tissues were sectioned by cutting perpendicular to the circular muscle layer at a thickness of 10–12 μm with a Leica CM 3050 cryostat (Leica Microsystems, Wetzlar, Germany). Sections were blocked with 1% BSA as described above before incubation with the first primary antibody. Following incubation in primary antibody (anti-GFP, Abcam; 1:1000 dilution in Triton-X working solution) for 16 h at 4°C, sections were washed with 0.1 M PBS before incubation in secondary antibody (Alexa Fluor anti-chicken 488, Invitrogen; 1:100 dilution). Subsequently sections were washed and blocked in 1% BSA in preparation for incubation with the second primary antibody [Alexa Fluor anti-goat 594 (Kit) or Alexa Fluor anti-rabbit 594 (smMHC), Invitrogen; 1:1000 dilution] as described above. After washing with PBS, slides were covered with coverslips using Aquamount mounting medium (Lerner Laboratories, Pittsburgh, PA, USA).
through a series of blunt pipettes with decreasing tip diameters in a final volume of ~1.5 mL.

**Cell sorting.** Dispersed cells were filtered through 100 μm mesh filters (Partec, Swedesboro, NJ, USA) and subsequently loaded onto a FACSaria II SORP System (BD Biosciences, San Jose, CA, USA). Green fluorescent protein-positive (GFP⁺) cells were sorted using a blue laser (488 nm) and the GFP/fluorescein isothiocyanate emission detector (530/30 nm band-pass and 505 nm low-pass) with a 130 μm nozzle at a sheath pressure of 12 p.s.i. and a sort rate of 200–800 events s⁻¹. Live cells were gated on the exclusion of Hoechst 33258 viability indicator dye and subsequently were gates on GFP fluorescence intensity. Sort counts were plotted graphically using FloJo software version 8.8.7 (Treestar, San Carlos, CA, USA).

**Gene expression**

**RT-PCR and qPCR on sorted cells.** Total RNA was isolated from sorted GFP⁺ cells and unsorted cells (cells taken from cell dispersions before sorting) using an illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Pittsburgh, PA, USA). First-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences) according to the manufacturer’s protocol. RT-PCR was performed with GoTaq DNA Polymerase (Promega) using the gene-specific primers listed in Table 1. PCR products were analysed on 2% agarose gels and were visualized using ethidium bromide. qPCR was performed using the primers listed in Table 1. Each sample contained tissues from two animals. All reactions were performed in technical duplicates.

**Contraction experiments**

Muscles for contractile experiments were prepared as previously described (Keef et al. 2013). IAS muscle strips (1 mm wide) were attached to a Gould strain gauge and a stable mount, immersed in warm (37°C) oxygenated (95% O₂/5% CO₂) KRBS and immediately stretched to achieve an initial force of 0.5 g. Thereafter, force declined to ~0.2 g before rising again with tone development (see Fig. 1). Tissues were equilibrated for 60 min before
beginning experiments. All experiments were carried out in the presence of atropine (1 μM), guanethidine (1 μM), Nω-nitro-L-arginine (L-NNA) (100 μM) and MRS2500 (1 μM) to eliminate the effects of spontaneous neurotransmitter release on the tissue. Maximum relaxation was determined by addition of the nitric oxide donor sodium nitroprusside (SNP, 10 μM) and the Cav1 blocker nifedipine (1–3 μM).

To evaluate the role of muscle stretch on tone development the protocol described by Zhang et al. (2016) was repeated. Briefly, tissues were dissected in cold (~4°C), oxygenated KRBS and mounted in a cold tissue bath in a ‘slack’ configuration (see Fig. 1A inset). Temperature was then raised to 37°C and the muscle equilibrated for 60 min. Thereafter, the muscle was stretched to achieve a peak force of 0.5 g.

Figure 1. Comparison of contractile activity in stretched versus un-stretched IAS muscle strips
The insert in A shows a diagram of the three treatment conditions for these experiments (Conditions 1–3). A, sample trace of the contractile activity occurring with Protocol 1. The solution was warmed from 4 to 37°C at the arrow. The muscle was equilibrated in a slack configuration (Condition 1) for 60 min in warm oxygenated KRBS. Stretch (St) to 0.5 g (Condition 3) was applied at the second arrow. The extent of active contraction was evaluated by addition of SNP (10 μM) and nifedipine (1 μM) at the third arrow. The amount of passive force was determined by reducing the separation of the wires (Unst, fourth arrow) until no further drop in force occurred. This value was equivalent to the initial baseline (Condition 1). B, sample trace of the contractile activity generated by a muscle mounted without stretch (Protocol 2, Condition 2). Phasic activity and tone developed in this muscle after the bath solution was warmed from 4 to 37°C (first arrow). In this example, stretch to 0.5 g was applied (Condition 3) after 60 min (second arrow). C, sample trace of the contractile activity generated by a muscle immediately stretched to 0.5 g (Protocol 3, Condition 3) following immersion in warm (37°C) oxygenated KRBS. Zero active force for this muscle (dotted line) was determined at the end of the experiment by addition of SNP (10 μM) and nifedipine (1 μM). D, summary graph of the amplitude of peak contraction, tone and phasic activity in stretched muscles (Protocol 3, Condition 3) versus un-stretched muscles (Protocol 2, Condition 2). Significantly greater force was generated for each component of contraction (#P < 0.05, n = 6) in stretched versus un-stretched muscles. Values shown are mean ± SEM.
(see Fig. 1A, Condition 3). Additional experiments were undertaken in which tissues were mounted in a straight but un-stretched configuration. This was achieved by increasing wire separation until the muscle was no longer bowed but before any force was registered on the force transducer. This second configuration is referred to as ‘un-stretched’ (see Fig 1A inset, Condition 2). Thereafter, the temperature in the tissue bath was raised to 37°C. In a few cases, after 60 min of equilibration, the un-stretched muscle was stretched to 0.5 g so that the subsequent contractile activity could be compared to that occurring when slack muscles were stretched to 0.5 g. A change in muscle length of ~40% was required to achieve a peak force of 0.5 g.

Contractile data were collected, stored and analysed by computer using AcqKnowledge software (3.9.1; Biopac Systems, Inc., Goleta, CA, USA). Concentration–response curves for blockers of contraction were determined by measuring the integral of the contractile trace (area) in the presence of each drug concentration and normalizing to the integral during control activity. The amplitude of peak contraction was determined by averaging all phasic contractile peaks during 60 s while tone was determined by averaging all trough values. Phasic contractile amplitude was derived by subtracting tone from peak contraction. Data sets for concentration–response relationships were fit with non-linear regression using GraphPad Prism Software (3.02; San Diego, CA, USA). IC50 values were obtained from these curves.

**Membrane potential experiments**

Muscle strips consisting of the final 2 mm of the GI tract were pinned submucosal side up to the base of a recording chamber and superfused with KRBS at 37°C. Cells located near the centre of the IAS (i.e. ~0.5 mm from the distal edge) were impaled with glass micro-electrodes filled with 3 M KCl (tip resistances 60–150 MΩ). To maintain impalements, tissues were initially bathed for 20 min in 20 μM wortmannin (myosin light chain kinase inhibitor) followed by a 45 min wash out period in regular KRBS before beginning recordings. The electrical events recorded in the presence of wortmannin [i.e. SW, spikes, resting membrane potential (Em)] are very similar to those recorded in the absence of wortmannin, while contraction is blocked (Duffy et al. 2012).

Membrane potential (Em) was collected using an Axoclamp 900A Microelectrode Amplifier and pCLAMP™10 Electrophysiology Data Acquisition and Analysis Software (Molecular Devices, Sunnyvale, CA, USA). To evaluate the effects of ion channel blockers on SWs, all values of Em (200 s−1) acquired during a 60 s recording period were used (12,000 total values, i.e. events). The total number of events at each consecutive 0.5 mV bin was derived with Origin50 software (OriginLab, Northampton, MA, USA) and plotted as ‘number of events vs Em’. A similar method has previously been used to evaluate spontaneous hyperpolarizations in the rat IAS (Opazo et al. 2011). ‘Resting’ Em was defined as the membrane potential associated with the greatest number of events. Because of the irregular nature of electrical activity, drug effects were quantified by multiplying each positive deviation above resting Em (e.g. ∆ Em = 0.5, 1, 1.5, 2, etc., mV) by the number of times this deviation occurred (i.e. number of events). These products were then summed together and divided by the total number of events positive to resting Em. This averaged value was then assigned the term ‘Average deviation positive to resting Em (mV).’

**Patch clamp experiments**

**Preparation of dispersed cells.** Small strips of IAS muscle were cut and prepared in Ca2+-free Hanks’ solution consisting of (in mM): 125 NaCl, 5.6 KCl, 15 NaHCO3, 0.36 Na2HPO4, 0.4 KH2PO4, 10 glucose, 2.0 sucrose and 10 Heps adjusted to pH 7.2 with Tris. After removing the mucosa, IAS muscles were equilibrated in the same solution for 30–40 min before enzyme treatment. Smooth muscles were exposed for 26 ± 2 min at 37°C to a solution containing (per ml) 4.0 mg collagenase (Worthington Type II; Worthington Biochemical), 8.0 mg BSA (Sigma), 8.0 mg trypsin inhibitor (Sigma), 250 μg ml−1 papain and 250 μg ml−1 diethioerythritol (both from Sigma). The digested tissue sections were rinsed briefly with Ca2+-free Hanks’ solution, and were gently triturated using a fire-polished glass Pasteur pipette to disperse the cells. Freshly isolated SMCs were used for patch clamp studies.

**Patch-clamp measurements.** Whole cell voltage-clamp experiments were performed on SMCs using the perforated patch technique, and amphotericin B (0.3 mg ml−1, Sigma-Aldrich) was dissolved in DMSO and diluted in the pipette solution with sonication. Membrane currents or transmembrane potentials were amplified with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), digitized with a 16-bit analog to digital converter (Digidata 1322A, Axon Instruments) and stored digitally using pCLAMP software (version 9.2, Axon Instruments). Data were sampled at 4 kHz and filtered at 2 kHz using an eight-pole Bessel filter. Mini-Digi with Axoscope (version 9.2, Molecular Devices) was used to monitor changes in holding currents (basal currents) throughout experiments. All data were analysed with Clampfit (version 10.5, Molecular Devices) and Graphpad Prism (version 6.0, Graphpad Software Inc., San Diego, CA, USA) software. Pipette tip resistances ranged between 2 and 3 MΩ and experiments were conducted at room temperature. External solutions were perfused and...
changed within 1 min with a fast bath perfusion system (AutoMate Scientific, Inc., Berkeley, CA, USA).

**Solutions for patch clamp experiments.** The external solution for whole-cell Cav_L recordings was a Ba^{2+}-containing salt solution (BaSS; mm): 135 NaCl, 5 KCl, 2 BaCl_2, 1.2 MgCl_2, 10 glucose and 10 Hepes adjusted to pH 7.4 with Tris. The pipette solution contained (mm): 135 CsCl, 3 MgATP, 0.1 NaGTP, 10 BAPTA, 10 gluicos, and 10 Hepes adjusted to pH 7.4 with Tris.

**Drugs**

Atropine sulphate, guanethidine, l-NNA, SNP and nifedipine were purchased from Sigma-Aldrich. Wortmannin was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). MR2500, T16Ainh-A01 and CaCCinh-A01 were purchased from Tocris Bioscience (Minneapolis, MN, USA). Atropine, guanethidine, l-NNA, MR2500 and SNP were dissolved in de-ionized water. Nifedipine was dissolved in ethanol. Wortmannin, T16Ainh-A01 and CaCCinh-A01 were dissolved in DMSO.

**Statistics**

Statistical analysis of multiple data sets was performed using one-way ANOVA followed by a post hoc Tukey multiple comparison test. Statistical analysis of two data sets was determined with a two-tailed unpaired Student’s t test. Data sets were considered significantly different at P < 0.05. Individual data points for graphs are expressed as mean ± SEM. N values indicate the number of animals except for qPCR experiments on isolated cells where n indicates the number of samples analysed with each sample containing tissues from 3–4 animals.

**Results**

**Role of stretch in the development of contraction in the IAS**

The role of stretch in tone development in the IAS was evaluated by comparing the contractile activity occurring with three different protocols. The first protocol was that used by Zhang et al. (2016). Muscles were mounted in a slack configuration (see Fig. 1A inset, Condition 1), immersed in a tissue bath and warmed to 37°C. After 60 min of equilibration muscles were stretched to 0.5 g (Condition 3). Zhang et al. (2016) reported that stretching muscles according to protocol 1 was followed by a rapid decline in force to a ‘lowest’ value. This was followed <5 min later by development of tone and phasic activity that reached maximum ~20 min after the initial stretch. Tone was defined as the difference between the ‘lowest value’ and peak contraction (see Zhang et al. 2016, suppFig.1). In contrast, when we repeated protocol 1 we found that phasic contractions and tone were immediately apparent after stretch (Fig. 1A, protocol 1), suggesting that this activity developed during the slack equilibration period. Thereafter, contractile activity slowly declined, reaching a steady state 15–20 min later. This protocol was repeated in nine muscle strips and in each case the same pattern occurred, i.e. we never observed an initial rapid drop in force to a ‘lowest value’ followed <5 min later by the development of tone and phasic activity. In the example shown in Fig. 1A, SNP and nifedipine were added 20 min after application of stretch to confirm that both phasic activity and tone were present in this tissue. Subsequent reduction of the wire separation (Unst) revealed a small component of passive force.

The second protocol undertaken examined whether active force develops in the absence of stretch. This was achieved by mounting the muscle and increasing wire separation until the muscle was no longer bowed but before any force was registered on the force transducer (Condition 2). In this configuration it was possible for the muscle to pull on the transducer wires if active force developed. After adjusting the wires in cold KRBS (4°C) the bathing solution was raised to 37°C (see Fig. 1B). Phasic activity and tone developed over the next 29.7 ± 1.8 min (Fig. 1B, n = 6). These data indicate that stretch is not required for tone development in the mouse IAS. When stretch was applied to these muscles after 1 h equilibration the subsequent contractile pattern was the same as that seen following stretch with protocol 1 (n = 4, Fig. 1A and B), i.e. we never observed an initial rapid drop in force to a ‘lowest value’ followed <5 min later by the development of tone and phasic activity.

The third protocol undertaken was the one used for all subsequent contractile measurements in this study, as well as previous studies by our lab (e.g. Keef et al. 2013). Dissected tissues were mounted and immersed in warm (37°C) oxygenated KRBS, immediately stretched to achieve an initial force of 0.5 g and then equilibrated for 60 min. When protocol 3 was employed, an initial rapid drop in force to a ‘lowest value’ was observed followed by the development of tone and phasic activity. Contraction reached a steady state 27.6 ± 2.9 min (n = 6) after the initial stretch (Fig. 1C). The time course for tone development using protocol 3 was not different (P > 0.05) from that observed using protocol 2. Once a steady state of contractile activity was attained it persisted for many hours. The ‘lowest value’ reached following stretch using this protocol was not zero tone. Rather zero tone (as determined by addition of SNP and nifedipine at the end of the experiment) was substantially less than this value (dotted line Fig. 1C).
Although tone development did not require stretch, the average amplitude of contractile components (i.e. peak, phasic, tone) was significantly less in un-stretched (Condition 2) versus stretched (Condition 3) muscles (Fig. 1D, n = 6) with total force (i.e. peak contraction) in un-stretched muscles being only 53.7% of that observed in stretched muscles. This observation is commensurate with the well-documented length–tension relationship of all muscles, including smooth muscle (e.g. Cooke & Fay, 1972). In support of this conclusion, we also found that when external K⁺ ([K⁺]₀) was raised by addition of 60 mM KCl to elicit a maximum contraction (Duffy et al. 2012) the response of un-stretched muscles was only 39.7% (0.57 ± 0.09 g, n = 6) of the response of stretched muscles (1.44 ± 0.08 g, n = 6).

**Effect of ANO1 and Cav₁ blockers on contraction**

The role of ANO1 on contractile activity in the IAS was investigated by determining the concentration-dependent effects of ANO1 antagonists, CaCCinh-A01 and T16Ainh-A01 (1–10 μM). These blockers caused near maximal inhibition of the phasic and tonic components of IAS contractions (Fig. 2A and B). The concentration–response curve for CaCCinh-A01 was shifted slightly to the left of that for T16Ainh-A01 (IC₅₀ = 2.2 and 3.9 μM, respectively; Fig. 2B).

The effect of stretch on ANO1 activation was also examined by comparing the responses to two CaCCinh-A01 concentrations on un-stretched muscles (Fig. 2C, Condition 2 of protocol 2) versus stretched muscles (Fig. 2A, Condition 3 of protocol 3). Inhibition of contraction was not different between these two conditions (P > 0.05; Fig. 2D). To evaluate possible non-specific effects, the same two concentrations of CaCCinh-A01 (3 and 10 μM) were also tested on contractions elicited by addition of 60 mM KCl. This resulted in an initial large transient contraction that declined to a much lower level. CaCCinh-A01 was then tested when a steady-state contraction was achieved (Fig. 2E). The potency of both CaCCinh-A01 concentrations on contractions induced by elevated [K⁺]₀ was significantly less (P < 0.05) than on spontaneous contractile activity. Specifically, spontaneous contraction was reduced 62.5 ± 5.4% by 3 μM CaCCinh-A01 (Fig. 2D) while contraction induced by elevated [K⁺]₀ was reduced by 4.2 ± 1.6% (n = 5). Likewise, spontaneous contraction was reduced 82.6 ± 4.8% by 10 μM CaCCinh-A01 (Fig. 2D) while contraction induced by elevated [K⁺]₀ was reduced by 13.8 ± 4.1% (n = 5).

The effect of the Cav₁ blocker nifedipine was also examined on spontaneous contractile activity. Nifedipine gave rise to a concentration-dependent inhibition of phasic activity and tone in the IAS (Fig. 3A). The IC₅₀ for this effect was 0.028 μM (Fig. 3B).

**Effect of a Cav₁ blocker on slow waves**

We previously characterized SWs in the mouse IAS (McDonnell et al. 2008; Duffy et al. 2012; Hall et al. 2014). In the present study, Eₘ was monitored to evaluate the effects of nifedipine and CaCCinh-A01 on SWs (Fig. 4A).

As previously reported and discussed (Duffy et al. 2012) the frequency of SWs recorded with a microelectrode in this study (49.9 ± 2.9 c.p.m., n = 11) was less (P < 0.05) than the frequency of phasic contractions recorded from the whole tissue (60.5 ± 3.1 c.p.m., n = 11). Nifedipine (0.03 μM) reduced SW amplitude significantly and transient depolarizations (TDs) appeared between SWs. Because of the irregular nature of this activity, the total number of times (events) that each value of Eₘ occurred during a 60 s recording was plotted as a function of Eₘ (see Methods). The range of potentials represented in this graph was greatest and the number of events for each potential was least during control SWs (Fig. 4C, closed circles). Following superfusion with 0.03 μM nifedipine, the range of potentials significantly decreased while the number of events for each potential increased (Fig. 4C, open squares). A further reduction in range and increase in number of events is seen with the highest concentration of nifedipine tested (1 μM; Fig. 4C, open circles).

In the IAS, a distinct period of quiescence between SWs is absent, making it difficult to assign a value for ‘resting’ Eₘ. For our purposes we have therefore defined resting Eₘ as the value at which the greatest number of events occurs (see Fig. 4C). The value of resting Eₘ attained with this procedure is shown as the dashed line in Fig. 4A. To evaluate how various treatments affected the overall occurrence of depolarizing events (i.e. SWs and TDs), all Eₘ events positive to resting Eₘ were averaged. In Fig. 4D, the ‘average deviation positive to resting Eₘ’ derived in this manner is plotted for various treatments. Control SWs had the greatest deviation whereas this deviation significantly declined with either 0.03 or 0.1 μM nifedipine. The average deviation with 0.1 μM nifedipine was not different from 1 μM nifedipine.

Nifedipine also depolarized resting Eₘ. This depolarization (Δ Eₘ) is illustrated in Fig. 4E. The average depolarization occurring with 0.03–1 μM nifedipine is summarized in Fig. 4F.

**Effect of ANO1 blockers on slow waves**

The effects of the ANO1 blocker CaCCinh-A01 was tested on SWs recorded from IAS muscles. CaCCinh-A01 caused a concentration-dependent reduction in SWs (Fig. 5A). In contrast to nifedipine, 3 μM CaCCinh-A01 was associated with substantial variation in amplitude between consecutive SWs. Nonetheless, the overall effect of 3 μM CaCCinh-A01 was to decrease (P < 0.05) the frequency of SWs (49.4 ± 3.6 c.p.m. versus 38.6 ± 2.9 c.p.m.,
Role of ANO1 in IAS

$n = 6$), decrease the range of membrane potentials represented and increase the number of events at each potential (Fig. 5B). SWs were abolished with $10 \mu M$ CaCCinh-A01. The effects of a different ANO1 blocker (T16Ainh-A01; $10 \mu M$) were very similar to those of CaCCinh-A01 (Fig. 5C). Both antagonists also produced a significant decrease in average deviation positive to resting $E_m$ (Fig. 5D) as well as a small but significant hyperpolarization of resting $E_m$ (Fig. 5E).

Effect of CaCCinh-A01 in the presence of nifedipine

As described above, Cav$_1$ and ANO1 blockers substantially reduced the average deviation positive to resting $E_m$.

![Figure 2. Comparison of two ANO1 blockers on contractile activity in the IAS](image)

A, sample trace showing the concentration-dependent effects of CaCCinh-A01 on contractile activity in the IAS. A very small additional relaxation is seen following addition of 10 $\mu M$ sodium nitroprusside (SNP) and 1 $\mu M$ nifedipine (Nifed). B, graph summarizing the concentration-dependent effects of CaCCinh-A01 ($n = 7$) and T16Ainh-A01 ($n = 6$) in the IAS. There is a small but significant difference ($P < 0.05$) in these concentration–response curves. C, sample trace showing the effects of 3 and 10 $\mu M$ CaCCinh-A01 on contractile activity in un-stretched muscles (i.e. Condition 2, Fig. 1). No additional relaxation was noted with addition of SNP and nifedipine. D, summary graph comparing the % inhibition of contraction with 3 and 10 $\mu M$ CaCCinh-A01 in stretched and un-stretched muscles. There was no significant difference in the actions of this blocker at either concentration ($P > 0.05, n = 6$). Values shown in B and D are mean ± SEM. E, sample trace showing the effects of 3 and 10 $\mu M$ CaCCinh-A01 on a KCl-induced contraction added after a steady state contraction was obtained. The effect of these concentrations of CaCCinh-A01 are markedly less than that seen in A and C. Subsequent addition of 1 $\mu M$ nifedipine (Nifed) eliminated the remaining active force while SNP (10 $\mu M$) was without effect.
Electrical activity in the presence of 1 μM nifedipine consisted of TDs and transient hyperpolarizations (THs) that occurred at random (Fig. 6A, upper trace). Further addition of CaCCinh-A01 (10 μM) in the presence of 1 μM nifedipine led to near complete blockade of TDs (Fig. 6A, lower trace). This effect is apparent as a reduction in $E_m$ range (Fig. 6B) and a significant decrease in the average deviation positive to resting $E_m$ (Fig. 6C). The average positive deviation measured following removal of the electrode from the cell (pull out) represents electrode noise. These data indicate that TDs are due to activation of ANO1 (Fig. 6B, C). THs are blocked by apamin or the P2Y1 receptor antagonist MRS2500 (Gil et al. 2012; Opazo et al. 2011; Kito et al. 2014).

**Effect of ANO1 blocker on Cav$_L$ currents**

In addition to the known effects of T16Ainh-A01 and CaCCinh-A01 on ANO1 (Bradley et al. 2014; Davis et al. 2013; Sung et al. 2016), there is also evidence that these blockers can have non-specific effects on Cav$_L$ (Boedtkjer et al. 2015). To address this possibility patch clamp experiments were undertaken on isolated SMCs from the IAS of the *wildtype* mouse to evaluate the effect of CaCCinh-A01 on Cav$_L$ currents. CaCCinh-A01 at 3 μM had no significant effect on Cav$_L$ currents whereas 10 μM CaCCinh-A01 led to a 30% reduction in Cav$_L$ current (Fig. 7).

**Immunohistochemical comparison of ANO1 protein expression in the IAS and rectum**

Dual labelling immunohistochemistry was undertaken on whole mount preparations to evaluate the expression pattern of ANO1 within the muscularis externa of *wildtype* muscles. Intense Kit immunoreactivity (red) was noted in ICC-IM of the IAS and rectum (Fig. 8). Kit$^+$ cells in both regions also labelled intensely with anti-ANO1 antibody (green) whereas ANO1 labelling was not observed in the spaces surrounding ICC-IM where SMCs are located. The cellular co-localization of ANO1 and Kit in ICC-IM is apparent in the merged images (yellow).

Antibodies available for ANO1 are not ideal because non-specific binding occurred in connective tissue, particularly when cryostat sections were used. To address this problem we therefore completed additional studies using the *Ano1*+*egfp* mouse. ICC were identified with anti-Kit antibody in cryostat sections cut perpendicular to the circular muscle layer (red). In this configuration, spindle shaped ICC-IM appear as dots distributed throughout the circular muscle layer of the IAS (Fig. 9A) and rectum (Fig. 9B). A well-developed plexus of ICC is apparent at the myenteric edge of the rectal circular muscle layer (ICC-My) as well as a less well-developed plexus at the submucosal edge of the rectal circular muscle layer (ICC-SM). As described previously, ICC-My and ICC-SM disappear before reaching the distal end of the IAS, leaving only ICC-IM (Hall et al. 2014). The egfp signal from the *Ano1*+*egfp* mouse was enhanced with an anti-GFP antibody (green, Fig. 9) and is referred to as ‘ANO1-egfp’. The cellular co-localization of ANO1-egfp and Kit in all populations of ICC is apparent in the merged files (yellow, Fig. 9).

In other experiments double labelling of ANO1-egfp and SMCs using anti-smMHC antibody (red) was performed. As before, cells immunopositive for ANO1-egfp were observed within the circular muscle layer (ICC-IM) of the IAS (Fig. 10A) and rectum (Fig. 10B). smMHC labelling clearly revealed the location of circular SMCs in both regions. However, in merged files there was an absence of yellow, indicating that all ANO1-egfp$^+$ cells were smMHC-negative (Fig. 10).

**Comparison of Ano1 and Cacna1c expression in the IAS and rectum**

The overall gene expression levels of Kit, *Ano1* and *Cacna1c* were evaluated by quantitative RT-PCR.
(qPCR) of whole muscle extracts from IAS and rectum. Expression levels for these three genes were not significantly different between regions (Fig. 11).

**Relative gene expression levels of Ano1 and Cacna1c in interstitial cells and SMCs in the IAS**

The relative gene expression levels of *Ano1* and *Cacna1c* were also examined in specific cell populations.
dispersed enzymatically from the IAS and purified by fluorescence-activated cell sorting (FACS). ICC, SMCs and PDGFRα+ cells were isolated from Kit<sup>opGFP</sup>/+<sup>,</sup> smMHC<sup>Cre-egfp</sup> and Pdgfra<sup>egfp</sup> mice, respectively. Each cell population was evaluated for purity by determining expression levels of cell-specific genes [e.g. Kit (ICC marker gene), Myh11 (smMHC gene) and Pdgfra (PDGFRα+ cell marker)] (Fig. 1A). Kit was enriched in GFP+ cells isolated from the Kit<sup>copGFP</sup>/+ mouse, Myh11 was enriched in GFP+ cells isolated from the smMHC<sup>Cre-egfp</sup> mouse and Pdgfra was enriched in GFP+ cells isolated from the Pdgfra<sup>egfp</sup> mouse, indicating that appropriate cell populations were obtained by the FACS protocols used. PDGFRα+ cells were included in this analysis because a unique population of cells which express PDGFRα have recently been identified in the muscularis externa of the GI tract including the IAS (Iino <i>et al</i>. 2009; Kurahashi <i>et al</i>. 2011; Cobine <i>et al</i>. 2011). Ano1 expression levels were 26.5-fold greater in ICC than in SMCs whereas the levels of Ano1 in SMCs and PDGFRα+ cells were not

![Figure 5. Effect of ANO1 blockers on slow waves (SWs) in the IAS](image)

<i>A</i>, sample trace showing inhibition of SWs following addition of CaCCinh-A01 (top). Three traces from the same recording are shown at faster sweep speed below (indicated by numbers). Dotted line highlights how <i>E</i><sub>m</sub> in the presence of blocker compares to control. Control resting <i>E</i><sub>m</sub> is indicated by the lower value shown on the scale bar.<br>

<i>B</i>, plot showing the number of times (events) that <i>E</i><sub>m</sub> was within consecutive 0.5 mV bins between −50 and −17 mV from the recording shown in <i>A</i> under control conditions (△) and in the presence of either 3 μM (●) or 10 μM (•) CaCCinh-A01. Note that for each condition one value of <i>E</i><sub>m</sub> is associated with the greatest number of events (i.e. resting <i>E</i><sub>m</sub>) and that resting <i>E</i><sub>m</sub> shifts to the left with CaCCinh-A01.<br>

<i>C</i>, sample trace showing inhibition of SWs following addition of 10 μM T16Ainh-A01 (top). Three traces from the same recording are shown at faster sweep speed below (indicated by numbers). Dotted line highlights how <i>E</i><sub>m</sub> in the presence of blocker compares to control. Control resting <i>E</i><sub>m</sub> is indicated by the lower value shown on the scale bar.<br>

<i>D</i>, summary graph of the average deviation positive to resting <i>E</i><sub>m</sub>. A significant reduction in average deviation (*<i>P</i> < 0.05) was observed with 3 μM CaCCinh-A01 (n = 4) versus control (n = 10) and a further reduction (*<i>P</i> < 0.05) occurred with 10 μM CaCCinh-A01 (n = 6). T16Ainh-A01 was also associated with a significant reduction (†<i>P</i> < 0.05, n = 9) in average deviation positive to resting <i>E</i><sub>m</sub>.<br>

<i>E</i>, summary graph of the amplitude of hyperpolarization with CaCCinh-A01 (left) or T16Ainh-A01 (right). There was no significant difference between antagonists. Values shown are mean ± SEM.
significantly different ($P > 0.05$, Fig. 12B). In contrast, the relative expression levels of Cacna1c transcript was SMCs > ICC = PDGFRα+ cells (Fig. 12C). The low expression levels of Cacna1c in IAS cells (≤6% of Gapdh) is similar to that of mouse colon where Cacna1c in SMCs is 7% of Gapdh expression levels (Lee et al. 2015).

**Discussion**

The present study shows that ANO1 and CavL play a central role in the generation of SWs, phasic contractions and tone in the IAS and that this pathway can occur in the absence of stretch. ICC-IM are the cells with predominant expression of ANO1, making them the most likely candidate for SW generation. The evidence in support of these findings is discussed below.

**Characteristics of SWs and conduction between cells in the IAS**

SWs have long been recognized as a fundamental electrical event timing phasic contractions in the GI tract (Szurszewski, 1987; Huizinga et al. 2009; Sanders et al. 2014). Typical SWs have a rapid upstroke (e.g. 434 mV s$^{-1}$ for canine antrum; Bayguinov et al. 2007) followed by a

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**Figure 6. Effect of ANO1 blockers on nifedipine-resistant transient depolarizations in the IAS**

A, sample traces from a single recording of $E_m$ in the presence of 1 μM nifedipine (top) followed by further addition of 10 μM CaCCinh-A01 (bottom). Dotted line indicates resting $E_m$. B, plot of the number of times (events) that $E_m$ was within consecutive 0.5 mV bins ranging between −50 and 0 mV for the recordings shown in A. Shown are the number of events in the presence of 1 μM nifedipine (-) with further addition of 10 μM CaCCinh-A01 (●) and following pull out of the electrode (□). Note that for each condition one value of $E_m$ is associated with the greatest number of events (i.e. resting $E_m$). C, summary graph of the average deviation positive to resting $E_m$. The amplitude of average deviation in the presence of nifedipine (n = 5) was significantly reduced ($^*$ $P < 0.05$) following addition of CaCCinh-A01 (n = 5). When the electrode was removed from the cell there was a further reduction in average deviation ($^*$ $P < 0.05$, n = 5) with the remaining deviation due to electrode noise. Values shown are mean ± SEM.

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**Figure 7. Effect of CaCCinh-A01 on CavL currents**

Left, sample traces of CavL currents elicited upon stepping from −80 to 0 mV in the absence (black) or presence of either 3 μM (red, upper trace) or 10 μM (blue, lower trace) CaCCinh-A01. Right, summary graphs of peak CavL currents in the absence (open bar) and presence of CaCCinh-A01. CaCCinh-A01 (3 μM, red) was without effect ($P > 0.05$, n = 5 cells from 5 tissues) while 10 μM CaCCinh-A01 (blue) was associated with a 30% reduction in CavL current ($P < 0.05$, n = 5 cells from 5 tissues). Values shown are mean ± SEM.

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plateau phase. The rapid upstroke is due to activation of T-type Ca\(^{2+}\) channels and it is resistant to Cav\(_L\) blockers whereas the plateau phase is reduced (Ward & Sanders, 1992). In contrast, IAS SWs have a much slower rise time (10–80 mV s\(^{-1}\)) (Duffy et al. 2012). They are also highly sensitive to blockade of Cav\(_L\) (present study), suggesting that this conductance may have an important role in their generation. IAS SWs resemble those in other regions in being rhythmic in nature but the frequency of these events is greater [e.g. SW frequency at the distal end of the mouse IAS averages 70 c.p.m. (Hall et al. 2014) whereas they are 3 and 30 c.p.m. in the mouse gastric antrum and jejunum, respectively (Hwang et al. 2009)]. Finally, like other regions, there is evidence that IAS SWs underlie phasic contractions (Kubota et al. 1998; Todorov, 1982; McDonnell et al. 2008). In summary, IAS SWs probably play an important role in regulating contraction in the IAS. The unique properties of SWs in the IAS underlines the importance of understanding how they are generated to gain greater insight into how motility is regulated in this muscle.

SWs in other GI muscles are conducted from ICC to adjacent SMCs via gap junctions, resulting in coordinated activity of the muscle (Sanders et al. 2014). In a previous study we examined the degree to which cells in the IAS are electrically coupled to one another by simultaneously

![Figure 8. Immunohistochemical identification of ANO1 in ICC-IM of the IAS and rectum](image)

Images from whole mount preparations of *wildtype* mouse IAS (A) and rectum (B) are shown. Kit\(^+\) ICC populations (red) in both regions were also ANO1\(^+\) (green) as confirmed in the merged files (yellow).

![Figure 9. Immunohistochemical identification of ANO1-egfp in ICC populations of the Ano1\(^{+/+}\)egfp mouse IAS and rectum](image)

Cryostat sections cut perpendicular to the circular muscle (CM) layer. Kit\(^+\) ICC-IM (red) appear as dots distributed throughout the CM layer of the IAS (A) and rectum (B). A well-developed network of ICC is also apparent at the myenteric edge (ICC-My) of the rectal circular muscle layer along with a less well-developed network of ICC at the submucosal edge (ICC-SM) of the rectal CM layer. The ANO1-egfp signal in sections was amplified with an anti-GFP antibody (green). Dual labelling of sections revealed that only Kit\(^+\) cells expressed detectible levels of ANO1-egfp (merge; Ac, Bc). The edge of the IAS circular muscle layer is indicated with a white dotted line. A small segment of the IAS longitudinal muscle layer (LM) can be seen in the upper right corner.
recording electrical activity at two sites with dual micro-electrodes (Hall et al. 2014). This study revealed that 83% of dual SW recordings made in the oral direction had a constant phase relationship to one another (i.e. they were coordinated) for an electrode separation of 125 μm while 40% coordination was observed for an electrode separation of 250 μm. Even greater coordination was observed in the circumferential direction (i.e. 100 and 40% coordination with electrode separations of 125 and 1000 μm, respectively). Since the width of individual muscle bundles estimated from Masson’s Trichrome images averaged 134 μm, our results are compatible with extensive coupling between cells, at least within individual muscle bundles. Thus, the contractile activity of multiple cells in the IAS can be coordinated.

**Role of stretch in tone development**

A recent study of the mouse IAS reported that tone development in the IAS is due to the depolarization initiated by activation of ANO1 in SMCs. Since spontaneous activation of ANO1 currents was not observed in isolated SMCs, it was suggested that muscle stretch might provide the needed signal to produce a global rise in intracellular calcium (Zhang et al. 2016). Toward this end, the authors reported that tone developed over a 20–30 min period of time when slack muscles, equilibrated for 1 h in 37°C KRBS, were subsequently stretched (i.e. Protocol 1, Fig. 1A). Equilibrating tissues in 37°C KRBS following dissection in cold KRBS is important because during dissection there can be run down of ionic gradients that are maintained by temperature-dependent transporters (e.g. Na/K ATPase; Nakamura et al. 1999). Furthermore, during dissection the muscle may be exposed to substances released from injured cells that can inhibit contractile activity, and/or damaged cells that are coupled to other cells might cause depolarization of the muscle.

To investigate the role of stretch in tone development we repeated the protocol described previously (Zhang et al. 2016). When stretch was applied to slack muscles after 1 h of equilibration at 37°C (Fig. 1A, protocol 1), tone and phasic activity were immediately apparent, suggesting that this activity developed during the previous 60 min equilibration period. This conclusion is supported by our observation that tone and phasic activity develop shortly after immersing un-stretched muscles in a tissue bath and warming to 37°C (Fig. 1B). Thus, our data

![Figure 10](image-url)

**Figure 10. Immunohistochemical identification of smooth muscle myosin (smMHC) and ANO1-egfp in the muscularis of the Ano1+/egfp mouse IAS and rectum.**

Cryostat sections cut perpendicular to the circular muscle (CM) layer. The shape of the CM layer is readily apparent from the labelling pattern of smMHC (red) in both the IAS (A) and the rectum (B). A bright population of ANO1-egfp-expressing cells (green) is also present in the CM layer of the IAS and rectum as well as two additional populations in the myenteric and submucosal plexus regions (MP and SP, respectively). However, there was no apparent overlap in these cell populations as seen in the merged files (Ac, Bc) indicating that all ANO1-egfp+ cells were smMHC negative.
support the idea that tone and phasic activity develop in IAS muscles following restoration of physiological temperatures because of the re-establishment of ionic gradients and electrical coupling between cells and that stretch is not required. Furthermore, since CaCCinh-A01 was equally effective at blocking tone in stretched and un-stretched muscles it is clear that ANO1 can be activated in the absence of stretch.

In contrast to our observations with protocol 1, Zhang et al. (2016) reported that stretch after 1 h equilibration was followed by an immediate decline in force to a ‘lowest value’ followed <5 min later by development of tone and phasic activity that reached a steady state ~20 min later. Tone was defined as the difference between this ‘lowest value’ and the subsequent steady state (see, Zhang et al. 2016, supfig.1). The authors assigned zero to the ‘lowest value’ when comparing tone between two conditions (e.g. control versus Tnem16αSmko mouse) and then plotted the rise of force thereafter. Our studies examining protocols 1–3 suggest that this method has a number of problems. First, we find that tissues immediately stretched (i.e. protocol 3) have substantial tone below the ‘lowest value’ (see Fig. 1C). This tone will not be accounted for with the method described by Zhang et al. Second, since we did not observe an initial ‘lowest value’ with protocol 1 it would be impossible to quantify tone according to their method. Third, since we observed a ‘lowest value’ with protocol 3 but not with protocol 1, there are clearly time-dependent changes occurring in this muscle that make quantifying tone according to their method problematic.

The spontaneous contractile amplitude in un-stretched muscles was significantly less (i.e. 54%) than that of stretched muscles. Since muscle length was ~40% greater in stretched versus un-stretched muscles, we have suggested that the amplitude of contraction was greater because of the length–tension (aka length–force) relationship (Cooke & Fay, 1972). In support of this conclusion we also found that the contraction elicited when [K+]o was raised by 60 mm was significantly less in un-stretched muscles (i.e. 39.4%) than in stretched muscles. Raising [K+]o has been used by others to evoke an ANO1-independent contraction (Davis et al. 2013; Boedtkjer et al. 2015) because this manoeuvre depolarizes SMCs to near the chloride equilibrium potential (Aickin & Brading, 1982; Bulley & Jaggar, 2014). The fact that CaCCinh-A01 (3 and 10 μM) had minimal effect on contractions induced by raising [K+]o (i.e. 4 and 13.8%, respectively; Fig. 2E) provides further support for this assumption, particularly when one considers that 10 μM CaCCinh-A01 reduces CavL currents by 30% (Fig. 7).

On a functional level, it is difficult to understand how stretch could be a requirement per se in tone development in the IAS because this muscle remains contracted most of the time to fulfil its role in the maintenance of faecal continence.

Role of CavL and ANO1 in the generation of SWs and tone

CavL antagonists have previously been shown to inhibit contractile and electrical activity in the IAS (Cobine et al. 2007; Cook et al. 1999). The present study shows the highly potent nature of CavL block with an IC50 of 0.03 μM for contraction (Fig. 3B) and a two-third reduction in SW activity with 0.03 μM nifedipine (Fig. 4D). Thus, CavL clearly plays a prominent role in SWs, phasic contraction and tone generation in the IAS. ANO1 antagonists also blocked SWs and contractions, suggesting a prominent role for this conductance in both events as well. However, a complicating factor in these experiments is that ANO1 inhibitors have been shown to have blocking effects on CavL (Boedtkjer et al. 2015). We tested whether CaCCinh-A01 has non-specific effects upon CavL by performing patch clamp studies on CavL currents recorded in isolated SMCs of the IAS. These experiments revealed that 3 μM CaCCinh-A01 was without effect whereas, as noted above, 10 μM CaCCinh-A01 reduced currents by ~30%. Thus, our patch clamp data suggest that the effects of 3 μM CaCCinh-A01 on SWs and contraction are unlikely to be due to inhibition of CavL but rather to blockade of ANO1.

ANO1 and CavL antagonists nearly abolished IAS contraction, but there was a small difference in their effects on SWs. SWs were abolished by CaCCinh-A01, but TDs remained following blockade of CavL with nifedipine. Since 0.1 and 1 μM nifedipine were equally effective at inhibiting SWs, this indicates that 1 μM nifedipine was sufficient to produce complete blockade of CavL. Applying CaCCinh-A01 in the presence of 1 μM nifedipine eliminated TDs, suggesting that these events are due...
to periodic activation of ANO1 currents. The ability of CaCCinh-A01 to block TDs in the absence of CavL reinforces the idea that CaCCinh-A01 has effects unrelated to CavL.

Previous patch clamp and Ca\(^{2+}\) imaging studies of ICC in the mouse jejunum suggest that stochastic Ca\(^{2+}\) release events from cellular stores activate ANO1 currents (Zhu et al. 2015; Baker et al. 2016), resulting in spontaneous transient inward currents (STICs). STICs cause spontaneous transient depolarizations that activate voltage-dependent Ca\(^{2+}\) channels in pacemaker ICC. Ca\(^{2+}\) entry through these channels can cause synchronous activation of ANO1 channels and the generation of SW currents (Zhu et al. 2009, 2015; Lees-Green et al. 2014). The microelectrode voltage recordings performed in the present study are compatible with this general concept, suggesting a similar relationship exists between ANO1 and CavL in ICC-IM of the IAS and this may be the basis for SW generation in these muscles.

An additional effect of CavL and ANO1 blockers was that they caused small changes in \(E_m\). Nifedipine depolarized cells by \(-3–5\) mV, and ANO1 blockers hyperpolarized cells by \(-2–4\) mV. Ca\(^{2+}\) entry via CavL in SMCs has been linked to activation of large conductance Ca\(^{2+}\)-activated K\(^+\) channels (BK) in SMCs (Greenwood & Leblanc, 2007; Rothenberg, 2012), including those of the GI tract (Bayguinov et al. 2000). Thus, it is possible that nifedipine-induced depolarization reflects a similar relationship between CavL and BK in the IAS. In contrast, the hyperpolarization observed with CaCCinh-A01 and T16Ainh-A01 suggests that ANO1 makes a small contribution to resting \(E_m\) in the IAS. Since the predominant expression of ANO1 is in ICC-IM (Fig. 12), this is an example of how ICC tune the excitability of the integrated syncytium of SMCs, ICC and PDGFR\(\alpha^+\) cells (SIP syncytium), as previously suggested (Sanders et al. 2014). An interesting additional observation was that hyperpolarization occurred subsequent to the reduction in SW amplitude and frequency (see Fig. 5), indicating that hyperpolarization \textit{per se} cannot account for the inhibition of SWs.

Expression levels of ANO1 (encoded by \textit{Ano1}) and Cav1.2 (encoded by \textit{Cacna1c})

ANO1 and CavL currents are fundamental to the generation of SWs, phasic contractions and tone in the IAS. We therefore sought to localize where these key signalling proteins are expressed within the muscularis externa of the IAS. Immunohistochemistry showed robust labelling of ANO1-egfp\(^+\) cells that were confirmed to be ICC in the IAS and rectum, but ANO1 expression was never resolved in SMCs (labelled with anti-smMHC antibody). In addition, qPCR of whole muscles revealed that...
Ano1 expression levels in the IAS and the rectum were the same; this observation is inconsistent with the presence of a unique population of SMCs in the IAS that express ANO1 (Zhang et al. 2016). Furthermore, qPCR of FACS sorted cells showed that Ano1 expression in SMCs was only 3.8% of that in ICC. Since Kit expression in SMCs was similar to this (i.e. Kit in SMCs was 3.7% of that in ICC, Fig. 12A) these expression levels may simply reflect the degree of purity of SMCs and ICC achieved with FACS. Ano1 expression in ICC (i.e. 61% of Gapdh) was also much greater than Ano1 expression in whole muscles (i.e. 2.4% of Gapdh). This difference can be accounted for if one takes into consideration the estimated volume occupied by ICC in the whole tissue, i.e. ~5% (Cobine et al. 2011). When this estimate (5%) is used along with the expression levels of Ano1 in isolated ICC (61%), the predicted level of Ano1 expression in whole tissue is: 0.61 × 0.05 = 0.03 or 3% of Gapdh. This percentage is within the SEM of the value actually observed for Ano1 expression in whole tissue (i.e. 2.4% of Gapdh), again suggesting that Ano1 expression is limited to ICC. These data do not support the conclusion that a unique population of SMCs in IAS expresses ANO1 and that currents generated by this conductance are responsible for the generation of tone (Zhang et al. 2016). Instead, our expression studies indicate that ANO1 is almost exclusively restricted to ICC-IM. Our functional studies further suggest that activation of ANO1 in ICC-IM leads to generation of SWs followed by conduction to adjacent SMCs where phasic calcium entry through Cav1.2 sums to produce tone.

Experiments examining Cacna1c expression revealed equivalent levels of this gene in whole IAS and rectum while the expression pattern in FACS sorted cells indicated that Cacna1c was 2-fold greater in SMCs than in ICC. The greater expression of Cacna1c in SMCs is not surprising as calcium entry through this channel is the major signalling step involved in excitation–contraction coupling in most smooth muscles (Hofmann et al. 2014). On the other hand, Cacna1c expression in ICC along with the high sensitivity of SWs to Cav1.2 blockade suggests that Cav1.2 is the voltage-dependent conductance in ICC that entrains activation of ANO1 channels creating SWs. In fact, Cav1.2 channels, commonly known as high-threshold Ca2+ channels, are more suitable for providing voltage-dependent Ca2+ entry in pacemaker ICC in the IAS because of the relatively depolarized resting E_m of cells in this muscle (i.e. averaging ~45 mV; Hall et al. 2014). Cav3.2 channels, which are low-threshold, voltage-dependent Ca2+ channels involved in generation and propagation of SWs of the small intestine (Zheng et al. 2014), would be largely inactivated at the membrane potentials of cells in the IAS.

We also examined the expression of Ano1 in PDGFRα+ cells of the IAS because these cells are electrically coupled to SMCs and are part of the SIP syncytium (Sanders et al. 2014). Transcripts of Ano1 were barely detectible in PDGFRα+ cells and only ICC were ANO1-egfp+ in immunohistochemical studies. PDGFRα+ cells mediate purinergic transmission (Kurahashi et al. 2014; Baker et al. 2015) and patch clamp studies have never resolved voltage-dependent inward currents or Ca2+-activated Cl− currents in these cells (Sanders et al. 2014). These observations make PDGFRα+ cells the least likely member of the SIP syncytium to be the pacemaker cells responsible for SW generation in the IAS.

Differences in the functional properties of the IAS and rectum

In our study Ano1 and Cacna1c expression levels were not different in whole strips of the IAS and rectum. Thus, the question arises: why does tone develop in the IAS but not in the rectum? The answer is likely to depend upon the differences in SW activity in the two regions. SWs are greatest in amplitude and frequency at the distal end of the IAS and decline in the proximal direction (Mutafova-Yambolieva et al. 2003; Hall et al. 2014). Thus, tone occurs in the region of greatest SW activity. There are also differences in the classes of ICC present within the rectoanal region. In the rectum, stellate-shaped ICC are organized into a plexus at the myenteric (ICC-My) and submucosal (ICC-SM) edges of the circular muscle layer while a third population of spindle-shaped ICC are present within the musculature (i.e. ICC-IM). Both ICC-SM and ICC-My populations decline in the distal direction approaching zero at the end of the IAS. In contrast, ICC-IM are present throughout (Hall et al., 2014). In the large intestine SWs are generated by ICC-IM and ICC-My (Sanders et al. 2014). Since ICC-IM appear to assume the role as pacemakers in the IAS, it is likely that ionic conductances and/or Ca2+ release mechanisms also differ from ICC-IM in the rectum. Toward this end, we have already shown that the morphology of ICC-IM changes markedly from rectum to IAS in the monkey, proceeding from stellate-shaped cells in the IAS to spindle-shaped cells in the rectum (Cobine et al. 2010). Thus, the possibility that differences also exist in the functional properties of ICC-IM between regions is not without precedent. Indeed, previous studies of ICC-IM in the guinea-pig gastric corpus and pylorus suggest that ICC-IM can serve as pacemakers in the stomach (Hirst & Edwards, 2006; Van Helden & Imtiaz, 2003) whereas in the large intestine ICC-IM serve as mediators between nerves and SMCs (Sanders et al. 2014). The greater amplitude and frequency of SWs in the IAS may result in incomplete uptake of calcium between SWs leading to tone generation; this condition is somewhat analogous to the partial tetanus that occurs in skeletal muscles when stimulated at intermediate frequencies.
Other studies examining the role of ICC in generation of SWs in the mouse IAS

Previously we reported that Kit-labelling of ICC-IM was not resolved within muscle bundles of the IAS in \(W/W^r\) mice, whereas a small population of ICC remained along the submucosal edge of the muscle (ICC-SM; Cobine \textit{et al.} 2011). Later we found that SWs and phasic activity persisted in \(W/W^r\) mice IAS, leading us to suggest that ICC-SM were responsible for this activity (Duffy \textit{et al.} 2012). Recently we re-examined the spatial distribution of ICC populations more systematically and determined that: (1) only ICC-IM are present at the distal end of the IAS and (2) the amplitude and frequency of SWs is greatest at this end (Hall \textit{et al.} 2014). Thus, we revised our hypothesis to suggest that ICC-IM are the more likely candidate for SW generation even though this conclusion conflicts with our previous studies of the \(W/W^r\) mouse (Duffy \textit{et al.} 2012). The \(W/W^r\) mouse is not a quantitative Kit knockout since one of the Kit alleles (\(W\)) is null while the other results in a protein with reduced function (\(W^r\)) (Nocka \textit{et al.} 1990). Because of the incomplete knockout, Kit labelling of ICC throughout the GI tract is ‘patchy’ with Kit resolvable in some regions of tissue but not in others (Iino \textit{et al.} 2007). Consequently, the degree of ‘functional’ knockout can differ between regions (Sanders & Ward, 2007) and indeed even in different studies of the same region (Ward \textit{et al.} 1998; Zhang \textit{et al.} 2010). Since Kit knockout is incomplete in \(W/W^r\) mice, it is possible that functional ICC-IM remain in the IAS of these mice, although Kit protein is not resolved by immunohistochemistry. Alternatively, since gene differences in the \(W/W^r\) mouse are constitutive, it is possible that remodelling occurs to compensate for loss of ICC in the IAS. Such compensation might include gain-of-function for SMCs. These issues suggest that \(W/W^r\) mice are not ideal for examining the role of ICC-IM in the IAS. Therefore, in the present study we sought to identify unique conductances expressed by pacemaker cells (hypothesized to be ICC-IM) that could be blocked pharmacologically to determine the source of SWs in the IAS.

Differences in myofilament sensitivity versus electrical activity

This study focuses upon the role of ANO1 and Cav\(_1\) in the regulation of SWs and tone in the IAS. In contrast, studies by others have suggested that tone in the IAS is due to the greater sensitivity of the myofilaments to Ca\(^{2+}\) (Patel & Rattan, 2006). Our study does not discount possible differences in myofilament sensitivity between regions in the GI tract but it bears emphasizing that Cav\(_1\) inhibitors produce near complete blockade of contractile activity in the IAS. Thus, understanding the mechanisms underlying SW generation in the IAS and the changes in \(E_{\text{rev}}\) that occur during nerve-mediated responses (Cobine \textit{et al.} 2014) is fundamental to understanding how tone is regulated in this muscle.

In conclusion, our results suggest a central role for ANO1 and Cav\(_1\) in the generation of SWs, phasic contractions and tone in the IAS and that stretch is not required for tone generation. ICC-IM are the predominant cell type expressing ANO1 and are therefore the most likely candidate for SW generation. SWs appear to arise via the activation of ANO1 and Cav\(_1\) in ICC-IM and excitation–contraction coupling occurs via conduction of SWs to SMCs and Ca\(^{2+}\) entry via Cav\(_1\), resulting in phasic activity that sums to produce tone.

References

Aickin CC & Brading AF (1982). Measurement of intracellular chloride in guinea-pig vas deferens by ion analysis, chloride efflux and micro-electrodes. \textit{J Physiol} \textbf{326}, 139–154.

Baker SA, Drumm BT, Saur D, Hennig GW, Ward SM & Sanders KM (2016). Spontaneous Ca\(^{2+}\) transients in interstitial cells of Cajal located within the deep muscular plexus of the murine small intestine. \textit{J Physiol} \textbf{594}, 3317–3338.

Baker SA, Hennig GW, Ward SM & Sanders KM (2015). Temporal sequence of activation of cells involved in purinergic neurotransmission in the colon. \textit{J Physiol} \textbf{593}, 1945–1963.

Bayguinov O, Hagen B, Bonev AD, Nelson MT & Sanders KM (2000). Intracellular calcium events activated by ATP in murine colonic myocytes. \textit{Am J Physiol Cell Physiol} \textbf{279}, C126–C135.

Bayguinov O, Ward SM, Kenyon JL & Sanders KM (2007). Voltage-gated Ca\(^{2+}\) currents are necessary for slow-wave propagation in the canine gastric antrum. \textit{Am J Physiol Cell Physiol} \textbf{293}, C1645–C1659.

Boedtkjer DM, Kim S, Jensen AB, Matchkov VM & Andersson KE (2015). New selective inhibitors of calcium-activated chloride channels - T16A(inh) -A01, CaCC(inh) -A01 and MONNA – what do they inhibit? \textit{Br J Pharmacol} \textbf{172}, 4158–4172.

Bradley E, Fedigan S, Webb T, Hollywood MA, Thornbury KD, McHale NG & Sergeant GP (2014). Pharmacological characterization of TMEM16A currents. \textit{Channels (Austin)} \textbf{8}, 308–320.

Bulley S & Jaggar JH (2014). Cl\(^-\) channels in smooth muscle cells. \textit{Pflugers Arch} \textbf{466}, 861–872.

Chen H, Ordog T, Chen J, Young DL, Bardsley MR, Redelman D, Ward SM & Sanders KM (2007). Differential gene expression in functional classes of interstitial cells of Cajal in murine small intestine. \textit{Physiol Genomics} \textbf{31}, 492–509.

Cobine CA, Fong M, Hamilton R & Keef KD (2007). Species dependent differences in the actions of sympathetic nerves and noradrenaline in the internal anal sphincter. \textit{Neurogastroenterol Motil} \textbf{19}, 937–945.

Cobine CA, Hennig GW, Bayguinov YR, Hatton WJ, Ward SM & Keef KD (2010). Interstitial cells of Cajal in the cynomolgus monkey rectoanal region and their relationship to sympathetic and nitrergic nerves. \textit{Am J Physiol Gastrointest Liver Physiol} \textbf{298}, G643–G656.
Cobine CA, Hennig GW, Kurahashi M, Sanders KM, Ward SM & Keef KD (2011). Relationship between interstitial cells of Cajal, fibroblast-like cells and inhibitory motor nerves in the internal anal sphincter. Cell Tissue Res 344, 17–30.

Cobine CA, Sotherton AG, Peri LE, Sanders KM, Ward SM & Keef KD (2014). Nitricergic neuromuscular transmission in the mouse internal anal sphincter is accomplished by multiple pathways and post-junctional effector cells. Am J Physiol Gastrointest Liver Physiol 307, G1057–G1072.

Cook TA, Brading AF & Mortensen NJ (1999). Differences in contractile properties of anorectal smooth muscle and the effects of calcium channel blockade. Br J Surg 86, 70–75.

Cooke PH & Fay FS (1972). Correlation between fibre length, ultrastructure, and the length–tension relationship of mammalian smooth muscle. J Cell Biol 52, 105–116.

Davis AJ, Shi J, Pritchard HA, Chadha PS, Leblanc N, Vasilikostas G, Yao Z, Verkman AS, Albert AP & Greenwood IA (2013). Potent vasorelaxant activity of the TMEM16A inhibitor T16A(nih) - A01. Br J Pharmacol 168, 773–784.

Duffy AM, Cobine CA & Keef KD (2012). Changes in neuromuscular transmission in the WiW mouse internal anal sphincter. Neugastroenterol Motil 24, e41–e55.

Gil V, Gallego D, Moha Ou MH, Peyronnet R, Martinez-Cutillas M, Heruteaux C, Borsotto M & Jimenez M (2012). Relative contribution of SKca and TREK1 channels in purinergic and nitricergic neuromuscular transmission in the rat colon. Am J Physiol Gastrointest Liver Physiol 303, G412–G423.

Gomez-Pinilla PJ, Gibbons SJ, Bardsley MR, Lorincz A, Pozo MJ, Pasricha PJ, Van de Rijn M, West RB, Sarr MG, Kendrick ML, Cima RR, Dozois EJ, Larson DW, Ordog T & Farrugia G (2009). Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. Am J Physiol Gastrointest Liver Physiol 296, G1370–G1381.

Greenwood IA & Leblanc N (2007). Overlapping pharmacology of Ca2+-, Cl−- and K+-activated Cl− channels. Trends Pharmacol Sci 28, 1–5.

Hall KA, Ward SM, Cobine CA & Keef KD (2014). Spatial organization and coordination of slow waves in the mouse anorectum. J Physiol 592, 3813–3829.

Hirst GD & Edwards FR (2006). Electrical events underlying organized myogenic contractions of the guinea pig stomach. J Physiol 576, 659–665.

Hofmann F, Flockerzi V, Kahl S & Wegener JW (2014). L-type Ca2+1.2 calcium channels: from in vitro findings to in vivo function. Physiol Rev 94, 303–326.

Horiguchi K, Keef KD & Ward SM (2003). Distribution of interstitial cells of Cajal in tunica muscularis of the canine rectoanal region. Am J Physiol Gastrointest Liver Physiol 284, G756–G767.

Huang F, Zhang H, Wu M, Yang H, Kudo M, Peters CJ, Woodruff PG, Solberg OD, Donne ML, Huang X, Sheppard D, F ah y JV, Wolters PJ, Hogan BL, Finkbeiner WE, Li M, Jan YN, Jan LY & Rock JR (2012). Calcium-activated chloride channel TMEM16A modulates mucin secretion and airway smooth muscle contraction. Proc Natl Acad Sci USA 109, 16354–16359.

Huizinga JD, Zarate N & Farrugia G (2009). Physiology, injury, and recovery of interstitial cells of Cajal: basic and clinical science. Gastroenterology 137, 1548–1556.

Hwang SJ, Basma N, Sanders KM & Ward SM (2016). Effects of new-generation inhibitors of the calcium-activated chloride channel anoctamin 1 on slow waves in the gastrointestinal tract 3. Br J Pharmacol 173, 1339–1349.

Hwang SJ, Blair PJ, Britton FC, O’Driscoll KE, Hennig G, Bayguinov YR, Rock JR, Harfe BD, Sanders KM & Ward SM (2009). Expression of anoctamin 1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. J Physiol 587, 4887–4904.

Iino S, Horiguchi K, Horiguchi S & Nojyo Y (2009). c-KIT-negative fibroblast-like cells express platelet-derived growth factor receptor alpha in the murine gastrointestinal musculature. Histochem Cell Biol 131, 691–702.

Iino S, Horiguchi S, Horiguchi K & Nojyo Y (2007). Interstitial cells of Cajal in the gastrointestinal musculature of W mutant mice. Arch Histol Cytol 70, 163–173.

Keef KD, Saxton SN, McDowall RA, Kaminski RE, Duffy AM & Cobine CA (2013). Functional role of vasoactive intestinal polypeptide in inhibitory motor innervation in the mouse internal anal sphincter. J Physiol 591, 1489–1506.

Kito Y, Kurahashi M, Mitsui R, Ward SM & Sanders KM (2014). Spontaneous transient hyperpolarizations in the rabbit small intestine. J Physiol 592, 4733–4745.

Kubota M, Suitsa S & Szurszewski JH (1998). Membrane properties and the neuro-effector transmission of smooth muscle cells in the canine internal anal sphincter. J Smooth Muscle Res 34, 173–184.

Kurahashi M, Mutafova-Yambolieva V, Koh SD & Sanders KM (2014). Platelet-derived growth factor receptor alpha-positive cells and not smooth muscle cells mediate purinergic hyperpolarization in murine colonic muscles. Am J Physiol Cell Physiol 307, C561–C570.

Kurahashi M, Zheng H, Dwyer L, Ward SM, Koh SD & Sanders KM (2011). A functional role for the ‘fibroblast-like cells’ in gastrointestinal smooth muscles. J Physiol 589, 697–710.

Lee MY, Park C, Berent RM, Park PJ, Fuchs R, Syn H, Chin A, Townsend J, Benson CG, Redelman D, Shen TW, Park JK, Miano JM, Sanders KM & Ro S (2015). Smooth muscle cell genome browser: enabling the identification of novel serum response factor target genes. PLoS One 10, e0133751.

Lees-Green R, Gibbons SJ, Farrugia G, Sneyd J & Cheng LK (2014). Computational modeling of anoctamin 1 calcium-activated chloride channels as pacemaker channels in interstitial cells of Cajal. Am J Physiol Gastrointest Liver Physiol 306, G711–G727.

McDonnell B, Hamilton R, Fong M, Ward SM & Keef KD (2008). Functional evidence for purinergic inhibitory neuromuscular transmission in the mouse internal anal sphincter. Am J Physiol Gastrointest Liver Physiol 294, G1041–G1051.

Mutafova-Yambolieva VN, O’Driscoll K, Farrelly A, Ward SM & Keef KD (2003). Spatial localization and properties of pacemaker potentials in the canine rectoanal region. Am J Physiol Gastrointest Liver Physiol 284, G748–G755.

Nakamura Y, Ohya Y, Abe I & Fujishima M (1999). Sodium–potassium pump current in smooth muscle cells from mesenteric resistance arteries of the guinea-pig. J Physiol 519, 203–212.
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Nocka K, Tan JC, Chiu E, Chu TY, Ray P, Traktman P & Besmer P (1990). Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W37, Wv, W41 and W. EMBO J, 19, 1805–1813.

Opazo A, Lecea B, Gil V, Jimenez M, Clave P & Gallego D (2011). Specific and complementary roles for nitric oxide and ATP in the inhibitory motor pathways to rat internal anal sphincter. Neurogastroenterol Motil, 23, e11–e25.

Patel CA & Rattan S (2006). Spontaneously tonic smooth muscle has characteristically higher levels of RhoA/ROK compared with the phasic smooth muscle. Am J Physiol Gastrointest Liver Physiol 291, G830–G837.

Ward SM (2012). The BK channel: a vital link between cellular calcium and electrical signalling. Protein Cell 3, 883–892.

Sung TS, O’Driscoll K, Zheng H, Yapp NJ, Leblanc N, Koh SD & Sanders KM (2007). Anoctamins and gastrointestinal smooth muscle excitability. Exp Physiol 97, 200–206.

Singh RD, Gibbons SJ, Saravanaperumal SA, Du P, Hennig GW, Eisenman ST, Mazzone A, Hayashi Y, Cao C, Stoltz GJ, Ordog T, Rock JR, Harfe BD, Szurszewski JH & Farrugia G (2014). Ano1, a Ca\(^{2+}\)-activated Cl\(^{-}\) channel, coordinates contractility in mouse intestine by Ca\(^{2+}\) transient coordination between interstitial cells of Cajal. J Physiol 592, 4051–4068.

Sung TS, O’Driscoll K, Zheng H, Leblanc N, Koh SD & Sanders KM (2015). Intracellular Ca\(^{2+}\) release from endoplasmic reticulum regulates slow wave currents and pacemaker activity of interstitial cells of Cajal. Am J Physiol Cell Physiol 308, C608–C620.

Opazo A, Lecea B, Gil V, Jimenez M, Clave P & Gallego D (2011). Specific and complementary roles for nitric oxide and ATP in the inhibitory motor pathways to rat internal anal sphincter. Neurogastroenterol Motil, 23, e11–e25.

Zheng H, Park KS, Koh SD & Sanders KM (2014). Expression and function of a T-type Ca\(^{2+}\) conductance in interstitial cells of Cajal of the murine small intestine. Am J Physiol Cell Physiol 306, C705–C713.

Zhu MH, Kim TW, Ro S, Yan W, Ward SM, Koh SD & Sanders KM (2009). A Ca\(^{2+}\)-activated Cl\(^{-}\) conductance in interstitial cells of Cajal linked to slow wave currents and pacemaker activity. J Physiol 587, 4905–4918.

Zhu MH, Sung TS, O’Driscoll K, Koh SD & Sanders KM (2015). Intracellular Ca\(^{2+}\) release from endoplasmic reticulum regulates slow wave currents and pacemaker activity of interstitial cells of Cajal. Am J Physiol Cell Physiol 308, C608–C620.

Additional information

Competing interests

The authors of this study have no competing interests.

Author contributions

All experiments were carried out in the laboratory of KDK. CAC: Concept and design of experiments. Collecting, analysis and interpretation of data. Drafting the article. EEH: Collection, analysis and interpretation of qPCR data. MHZ: Collection, analysis and interpretation of patch clamp data. HEL: Collection, analysis and interpretation of patch clamp and microelectrode data. JRR: Generation of Ano1\(^{+/+}\) mouse, critical evaluation of the manuscript. KMS: Concept of experiments, critical evaluation of the manuscript. SMW: Concept and design of experiments, critical evaluation of the manuscript. KDK: Concept and design of experiments. Collecting, analysis and interpretation of data. Drafting the article. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work. We ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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