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Calcium-activated chloride channels anoctamin 1 and 2 promote murine uterine smooth muscle contractility

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Abstract

Objective—To determine the presence of calcium activated chloride channels anoctamin 1 and 2 in human and murine uterine smooth muscle and evaluate the physiologic role for these ion channels in murine myometrial contractility.

Study Design—We performed reverse transcription polymerase chain reaction to determine if anoctamin 1 and 2 are expressed in human and murine uterine tissue to validate the study of this protein in mouse models. Immunohistochemical staining of anoctamin 1 and 2 was then performed to determine protein expression in murine myometrial tissue. The function of anoctamin 1 and 2 in murine uterine tissue was evaluated using electrophysiological studies, organ bath, and calcium flux experiments.

Results—Anoctamin 1 and 2 are expressed in human and murine USM cells. Functional studies show that selective antagonism of these channels promotes relaxation of spontaneous murine

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uterine smooth muscle contractions. Blockade of anoctamin 1 and 2 inhibits both agonist-induced and spontaneous transient inward currents and abolishes G-protein coupled receptor (oxytocin) mediated elevations in intracellular calcium.

Conclusion—The calcium activated chloride channels ANO 1 and 2 are present in human and murine myometrial tissue and may provide novel potential therapeutic targets to achieve effective tocolysis.

Keywords

anoctamin; calcium channels; contractility; myometrium; uterus

Introduction

Preterm birth (PTB) remains a major obstetric crisis affecting 1 in every 8 births in the US -- a value that has increased by approximately 36% since 1986 [1]. PTB is the leading cause of neonatal death in the US, accounting for 35% of deaths in the first year of life [2], and preterm infants that survive are at significantly increased risk for lifelong morbidities [3]. The CDC estimates that PTB associated morbidity results in $26 billion in healthcare expenditures in the US alone [1]. Etiologies of PTB vary, however one of the most common causes of PTB is spontaneous preterm labor (PTL) [3]. Although there have been recent advances in the prevention of PTB [4], treatment of spontaneous PTL with currently available tocolytics (magnesium, nifedipine, terbutaline, and indomethacin) is ineffective beyond 48 hours [5]. Therefore, identification of novel tocolytic strategies is necessary to prevent this debilitating obstetric problem. Our goal is to evaluate whether the calcium activated chloride channels anoctamin 1 and 2 (ANO 1 and 2) could be novel targets for effective tocolysis.

The organization of phasic uterine contractions during labor is hypothesized to depend on two processes. First, membrane depolarization causes voltage-gated calcium entry leading to an action potential (AP) and a subsequent intracellular calcium wave. Second, this AP is propagated intercellularly via gap junctions (connexin 43, for example) on myometrial cells [6], resulting in the spread of phasic, coordinated contractions throughout the uterus. Rhythmic uterine contractions are associated with oscillations in the membrane potential of myometrial cells. Membrane potential oscillations summate to cause “slow waves” which promote uterine APs and thus contractions [7]. Changes in membrane potential are achieved by coordinated actions of two classes of ion channels/transporters: those that exert hyperpolarizing currents (eg potassium channels and the NaK-ATPase pump), and those that exert depolarizing currents (eg calcium activated chloride channels and T-type calcium channels) [8].

Research into the functional roles of ANO 1 and 2 has exploded in recent years due to the discovery that these channels are responsible for the long observed physiologic calcium-activated chloride current [9, 10, 11]. These proteins recapitulate the properties known to be fundamental to endogenous calcium activated chloride channels (CaCCs), including chloride mediated current, voltage and calcium dependent activation [10], and decreased iodide flux in response to ANO 1 siRNA knockdown [9]. Subsequent work in various smooth muscle

beds has demonstrated that ANO 1 serves a fundamental role in organizing smooth muscle contraction. In particular, ANO 1 mediated chloride current has been shown to alter cell membrane depolarization and slow waves in the GI tract [12, 13], urethra [14], and oviduct [15]. Additionally, ANO 1 is functionally expressed in airway smooth muscle and its blockade attenuates contractions as well as pro-contractile membrane associated currents [16, 17]. To date, the physiologic role of ANO 1 and 2 in the human and murine myometrium remains unclear as studies evaluating these ion channels in myometrial cells and tissue are lacking.

Our goal was to evaluate whether human and murine USM cells express ANO 1 and 2 and to determine the physiologic role of these CaCCs on murine uterine smooth muscle (USM) contractility. In particular, we sought to determine whether murine USM cells demonstrate classic electrophysiological evidence of a voltage regulated outward rectifying calcium activated chloride current, whether pharmacologic blockade of ANO 1 and 2 relaxes spontaneousUSM contractions, and whether ANO 1 and 2 blockade attenuates contractile agonist-induced elevations in intracellular calcium.

Materials and Methods

All animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Materials

TRIzol® reagent was obtained from Ambion-Applied Biosystems (Austin, TX, USA). Conventional PCR and RT-PCR reagents were purchased from Clontech Laboratories (Mountain View, CA) and Applied Biosystems (Carlsbad, CA). Human USM cells and culture media were purchased from Lonza (Wakersville, MD). C57-Bl6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All other pharmaceutical drug reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

Cell Culture

Murine uterine smooth muscle (USM) cells were harvested for use in culture using an enzymatic cellular dissociation kit (Worthington, Lakewood, NJ) and grown in SmBMII media with the manufacturer's recommended additives (Lonza, SmGM-2 Singlequots Kit suppl & growth factors). Non-pregnant human USM cells were purchased from Lonza (CC-2562) and grown in SMBMII as above. Cells were grown to confluence in T75 cm^2 flasks in a humidified 37°C incubator in 95% air/5 % CO_2.

RNA extraction and reverse transcription of cDNA in murine and human USM

Total RNA was extracted from primary murine USM cells, flash frozen whole mouse brain (positive control), and cultured human USM cells using Trizol® according to the manufacturer's recommendations. Human brain total RNA was purchased from Clontech (#636530). RNA purity and quantity was measured by 260/280nm absorbance (Beckman Du 640). Total RNA from human and murine USM was diluted in RNAse free water to a final concentration 500 μg/ml, aliquoted and stored at −80°C. Complementary DNA synthesis

was performed (1μg total RNA in a final volume of 20 μl) with MMLV RT Reverse Transcriptase-Advantage® RT for PCR (Clontech) using either oligo (dT) or random hexamer priming following the manufacturer's recommendations.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Newly synthesized cDNA (5μl of original 20μl reaction) was used in PCR with the Advantage® 2 PCR Kit on an MJ Research PTC-200 Peltier thermal cycler (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Sense and antisense primers specific for ANO 1 and 2 were constructed using primer blast (Table 1) and were purchased from Invitrogen (Carlsbad, CA). GADPH was used as an internal control. Primers were designed so that amplicon size for mRNA and genomic DNA were easily distinguishable. cDNA samples were initially denatured at 94°C for 30s. The annealing temperatures for ANO 1 and 2 were set to 64°C and 68°C, respectively, based on previously obtained optimal temperatures [17]. For RT-PCR, there was a completion step of 30 seconds. PCR products were analyzed on 10% polyacrylamide gels stained with ethidium bromide (Molecular probes, Eugene, OR) and visualized using a gel imager and VisionWorks software (Biospectra UVP, Upland, CA).

Immunohistochemistry of murine USM for ANO 1 and 2 expression

Uterine smooth muscle was dissected from non-pregnant C57-bl6 mice (18-20 grams) and immediately fixed in 4% paraformaldehyde (4°C overnight), then incubated in 30% sucrose in PBS for an additional 24 hours prior to processing for cryostat sectioning. The sections (6 μm) were washed in PBS, incubated with 0.1% Triton X-100 for 10 min, blocked with 15% goat serum, then incubated overnight at 4°C in primary antisera. In order to confirm that protein expression persists in primary culture conditions, parallel experiments were conducted using primary murine USM cells. These cells were cultured on coverslips in SmBMII smooth muscle media, containing 5% FBS and other manufacturer recommended additives, for 2-3 days then fixed with 3% paraformaldehyde in PBS for 5 minutes. Primary antibodies used were (1) anti-ANO1 (rabbit, monoclonal; Abcam #ab64085, 1:100 dilution in PBS) for cultured cell immunocytochemistry, (2) anti-ANO1 (rabbit polyclonal; Abcam #ab53213, 1:1 dilution in PBS) for murine uterine tissue immunohistochemistry, and (3) Rhodamine Phalloidin for cultured cell actin cytoskeleton immunocytochemistry (1:100, Life Technologies, Grand Island, NY).

Secondary antibodies consisted of FITC-conjugated goat anti-rabbit IgG (1:400 dilution), incubated for 1hr. Nuclear staining was achieved employing mounting medium pre-mixed with DAPI stain (Vector laboratories, Burlingame, CA, H-1500). Negative controls were performed on serial sections by omitting primary antibody. All the immunofluorescence experiments were repeated at least 3 times. Samples were viewed under confocal microscopy (Nikon Eclipse, Japan) and images were acquired with NIS software version 4.10.

Immunoblot for ANO 1/2 protein expression

Mouse uterine primary cultured (MUSM) and human airway smooth muscle cells (HASM; positive control) were utilized for western blot following homogenization in ice-cold NP40

cell lysis buffer (50mM Tris, 250mM NaCl, 5 mM EDTA, 50mM NaF 1mM Na3VO4, 1% Nonide P40 and 0.02 % NaN_3). Following centrifugation (5000g, 5min, 4°C) of the whole cell lysate, the supernatants wer e saved and protein concentrations were determined. Aliquots were solubilized by heating at 95°C for 5 min in sample buffer, were subsequently electrophoresed through a 4 - 15% Mini-PROTEAN TGX gel (Bio-Rad), transferred to PVDF, and probed with TMEM16A antibody (rabbit polyclonal was diluted to 1:4, No. ab53213; Abcam, Cambridge, MA). Equivalent loading was confirmed by commensurate probing with β-actin (rabbit monoclonal diluted to 1:1,000, Millipore). The primary antibodies were detected by horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:3,000 for TMEM16A; 1:5,000 for β-actin). The signal from the immunoreactive bands was detected by enhanced chemiluminescence (SuperSignal West Femto; Thermo Scientific, no.34095, Rockford, IL) according to the manufacturer's recommendation. Digital acquisition of the resulting bands was achieved using UVP biochemi system (UVP, LLC, CA) and Vision Works LS software.

Patch clamp electrophysiological studies in murine USM cells

To assess membrane potential currents relevant to the physiology of contractility, we performed whole cell patch clamp studies. Murine USM was digested with collagenase type IV at 37C for 5-10 minutes. The released cells were placed on poly10 L-Lysine 12mm coverslips (BD, San Jose, CA), coated with 0.5 mg/ml collagen Type I (Sigma) and incubated for another 1-3 days. For whole cell recordings, coverslips were transferred to a 0.5 ml chamber on the stage of an inverted microscope (Nikon). Membrane currents were recorded using whole-cell configuration. The extracellular solution contained (in mM) 130.0 NaCl, 5.5 tetraethylammonium chloride (TEA-Cl), 2.2 $CaCl_2$, 1.0 $MgCl_2$, 10.0 HEPES, 10.0 Glucose, pH adjusted to 7.35 with NaOH. The pipette solution contained (in mM): 75.0 CsCl, 64.0 Cs-gluconate, 1.0 $MgCl_2$, 10.0 HEPES, 3.0 Na_2ATP, pH adjusted to pH 7.3 with CsOH. Whole-cell currents were recorded using Axopatch 200B coupled to a 1322A digitizer. The patch pipette had a resistance of 3-5 MΩ. All recordings were performed at room temperature. Criteria for quantifying spontaneous transient inward currents (STICs) included currents with anamplitude twice the baseline as detected with pCLAMP10 and analysis by Origin 8 software. A voltage ramp from −60V to 40V was performed and STICs were recorded to generate a voltage-current relationship. In parallel studies, the impact of selective ANO 1/2 antagonists on STICs were analyzed. Briefly, following recording of spontaneous STICs, 100uM benzbromarone, a powerful and selective ANO 1/2 antagonist was applied and STICs were measured. In addition, the effect of benzbromarone on GPCR and ryanodine receptor mediated activated STICs was also studied. All drugs used were reconstituted from stock solutions to final working concentration on the day of the experiment. Optimal concentrations were derived from preliminary dose response experiments [17]. Benzbromarone was dissolved in DMSO, the final concentration of which was less than 0.05%.

Murine USM organ bath contractility studies

USM tissue was harvested from euthanized C57-Bl6 mice. Five mm circular segments of intact USM were cut from the uterine horn above the bifurcation. Segments were cleaned from mesenteric attachments. Ringed segments were placed in 8ml organ baths (DMT

Tissue Organ Bath 720MO Danish Myo Technology, Denmark) in a modified Krebs-Henseleit buffer (concentration in mM: NaCl 115.0, KCl 2.5, $CaCl_2$ 1.9, $MgSO_4$ 2.5, $NaHCO_3$ 25.0, NaH_2PO_4 1.4, D-glucose 5.6). Muscle force was digitally recorded during all experiments using BioPac hardware and AcqKnowledge 3.7.3 software (Biopac Systems, Inc., Goleta, CA). Rings were equilibrated to 1.0 gram of isometric tension for 1 h with Krebs–Henseleit buffer, based on preliminary experiments performed to determine the optimal starling forces for organ bath contractility (data not shown). Buffer was warmed to 37°C, replaced every 20 minutes, and continuously bubbled with 95% O_2 / 5% CO_2. Following buffer exchanges and resetting of tension to 1.0 gram, rings were allowed to contract spontaneously. After reaching steady state for contractile frequency and amplitude, rings were then randomly treated with vehicle, tannic acid 100 μM (an ANO 1/2 selective antagonist) [18], or niflumic acid 100 μM (a ubiquitous chloride channel blocker was employed as a positive control). The 100 μM dose for tannic acid was chosen based on preliminary dose response organ bath studies that yielded an IC_{50} of 96 μM (data not shown). Following addition of these drugs, muscle force was analyzed over the next 30 minutes examining changes in mean amplitude of force, and frequency compared to both time matched and vehicle treated controls. The pH was measured and maintained at 7.4 throughout the experiment in both the control and treatment groups with no significant change noted after addition of tannic or niflumic acid. Drugs were made from stock solutions and diluted to final desired concentrations.

Calcium flux studies

All calcium studies were performed using the ratiometric fluorescent calcium indicator Fura-2 (Calbiochem, Billerica, MA) as previously described [19]. Murine USM cells were grown to 100% confluence in 96 well black-walled clear-bottom plates between passages 4-10. Cells were washed with modified HBSS (concentration in mM: NaCl 137.9, KCl 5.3, $CaCl_2$ 2.0, $MgSO_4$ 1.0, Hepes 2.4, glucose 5.5, pH to 7.4). The cells were then loaded with 100uL of 5uM Fura-2 AM in a humidified 37C incubator (95% air / 5% CO_2) for 30 minutes. Cells were washed again with HBSS and then incubated in HBSS for 20 minutes to allow for de-esterification of the indicator. Cells received pretreatments with either an ANO 1/2 specific antagonist (benzbromarone or tannic acid), a distinct ubiquitous chloride channel antagonist (5-Nitro-2-3-phenylpropylamino benzoic acid (NPPB)) was utilized as a positive control, or vehicle as a negative control for 10 minutes prior to study. Following this incubation, cells were exposed to either oxytocin or bradykinin to induce GPCR mediated calcium release. The experiment was performed bradykinin as a Gq coupled agonist to confirm that the chloride channel antagonists' effect was not limited to action via the oxytocin receptor. Fluorescence was measured in real time at 37C using a Flex Station 3 (Molecular Devices) using excitation wavelengths of 340 and 380, an emission wavelength of 510, and a cutoff filter of 495. Fluorescence values were reported as F/Fo according to the calculation:

$$[\Delta \mathrm{F} = (340 \quad nm)\,\mathrm{f}/\,(380 \quad nm)\,\mathrm{f} - (340 \quad nm)_0/(380 \quad nm)_0]$$

Peak values obtained following drug or vehicle administration were then examined for group comparisons. Benzbromarone was prepared in DMSO, the concentration of which never exceeded 0.1%.

Statistical Analysis

Electrophysiological measurements of current and frequency were analyzed by unpaired, two tailed t-tests. Organ bath and calcium studies were analyzed using one-way ANOVA with Bonferroni correction for intergroup statistical comparisons. The data are expressed as mean ± S.E.M. In all cases, a *P* value less than 0.05 was considered as statistically significant.

Results

Qualitative expression of mRNA encoding ANO 1 and 2 in primary murine and cultured human USM

Messenger RNA encoding ANO 1 and 2 was found in both in murine and human USM, with brain serving as a positive control (Figure 1A). Identification of ANO 1 mRNA expression in human cultured cells validates our study of this protein in a mouse model.

ANO 1 and 2 proteins are expressed on primary and cultured murine USM cells

To examine ANO 1 and 2 protein expression in the murine uterus, immunohistochemistry was performed on fixed permeabilized tissue. In addition, parallel experiments were performed to confirm that protein expression persists in primary cultured conditions in isolated cultured smooth muscle cells. Double staining for ANO 1/2 and actin shows ANO 1/2 expression on USM, and absence of ANO 1/2 from vascular smooth muscle (Fig. 1B. a, b, c, d).

STICs are present in the murine myometrium, are responsive to voltage, and reverse direction at the reversal potential for chloride

Experiments were carried out using a pipette solution primarily containing cesium chloride and bath solution containing tetraethylammonium to limit confounding potassium current effects. Under whole-cell voltage-clamp configurations, two types of spontaneous inward current patterns were evident. Using a holding potential of −60 mV, we observed both a continuous randomly generated inward current (Fig. 2a) as well as a distinct inward current characterized by spontaneous rhythm or auto rhythmicity (Fig 3C inset). Consistent with a calcium-activated channel, both of these currents were greatly diminished in the presence of a calcium free buffer (data not shown). The spontaneous rhythm demonstrates a period of STIC duration of 330 ± 13.6ms (n=10) followed by a 193.4 ± 9.8 ms (n=10) pause between adjacent current clusters. There was no noticeable difference in current amplitude between the two current patterns. The group of cells showing auto-rhythmicity comprised about 5% of the total number of cells recorded. The average amplitude and frequency of STICs from both rhythm patterns were −160.8 ± 97 (pA) and 5.2 ± 3 Hz, respectively (n=23, events 998). Fig.2a displays a representation of six cells in which STICs were recorded at different voltages. At −60 mV holding potential, the inward current was −200.2 ± 41.5 pA (n=6).

The calculated E_{Cl} is −13.8 mV. At potentials more negative than −13 mV, STICs were inward and at potentials more positive than the E_{Cl}, they changed direction from inward to outward, suggesting that STICs reverse at the reversal potential for chloride. At a holding potential of 40mV, the outward current was 291 ± 56 pA (n=6). Fig. 2b shows the relationship between averaged current amplitude and holding voltage.

Murine USM STICs are activated by calcium and blocked with ANO 1/2 antagonists

To investigate if ANO 1 and 2 channels contribute to STICs in murine USM cells, we tested the effects of the ANO 1/2 channel blocker benzbromarone and found that 100 μM benzbromarone rapidly attenuates baseline STIC activity, decreasing amplitude by 32% ± 1% (n=8) and frequency by 43% ± 14% (n=8) of control (Fig.3 Aa, 3 B).

Oxytocin regulates a large number of reproduction-related processes in all species. Particularly important is its ability to stimulate uterine contractility by mechanisms involving sarcoplasmic reticulum calcium release and sensitization of the contractile apparatus to calcium [20]. Application of 20 μM oxytocin stimulated STIC amplitude by 180% ± 44% (n=5) and frequency by 300% ± 44% (n=5) of control. The effect of oxytocin on STICs was inhibited by 100 μM benzbromarone, decreasing the effect of oxytocin on amplitude and frequency by 56 % ± 5% (n=5) and 55% ± 8% (n=5) of control, respectively. (Fig.3 Ab, 3 B).

To test STIC activation by increased intracellular calcium via another mechanism, sarcoplasmic calcium was liberated from ryanodine receptors by activation with caffeine. Fig. 3 Ac shows that 10 mM caffeine increased the amplitude and frequency of STICs by 176% ± 24% (n=5) and 210% ± 53% (n=5) of control respectively. The caffeine-mediated activation of STICs was inhibited by 100 μM benzbromarone, which decreased amplitude and frequency by 60 % ± 20% (n=5) and 51% ± 7% (n=5) of control, respectively (Fig. 3 Ac, 3 B).

ANO blockade inhibits spontaneous contractions in murine myometrial tissue

To investigate the effect of ANO 1 and 2 blockade on USM contractility, murine uterine rings were studied in the organ bath (Fig 4 A, B, C). Following the initiation of spontaneous contractions resulting after application of isometric tension (~1.0g), treatment with the ANO 1/2 selective antagonist tannic acid (100 μM) reduced contraction frequency to 28.28% ± 10.0% (N=5, $p < 0.001$) of vehicle control (100% ± 17%). As expected, treatment with 100μM niflumic acid (positive control) reduced contraction frequency to 80.76% ± 5.0% (N=5, $p < 0.01$). Selective ANO 1/2 blockade with 100 μM tannic acid reduced contraction frequency significantly more than ubiquitous chloride channel blockade with100 μM niflumic acid (Fig 4B right tracing; $p < 0.001$). 100 μM tannic acid and 100 μM niflumic acid both decreased contraction amplitude compared with control, to 65.32% ± 18.0% (N=5, $p < 0.001$) and 57% ± 10.0% (N=5, $p < 0.001$) respectively. The two chloride channel blockers did not differ significantly from each other in amplitude (Fig 4B, left tracing).

Contractile agonist-induced calcium elevation is inhibited under conditions of ANO 1/2 blockade

To elucidate the underlying mechanism by which ANO 1 and 2 blockade inhibits USM contractility, we examined GPCR-mediated intracellular calcium elevations using pretreatments with ANO 1/2 selective antagonists (benzbromarone or tannic acid), NPPB (positive control), or vehicle. A representative Fura-2 tracing is shown in Figure 5A. Both oxytocin (1μM) and bradykinin (10μM) elicited robust increases in intracellular calcium, with mean peak Fura-2 fluorescence (F/F_0) of 1.12 ± 0.13 (N=10) and 0.97 ± 0.12 (N=5) respectively. Cells that received pretreatment with benzbromarone produced significantly lower elevations in intracellular calcium response, with peak fluorescence of 0.1 ± 0.04 (N=10, $p < 0.001$) in cells receiving oxytocin and 0.1± 0.06 (N=5, $p< 0.001$) in cells receiving bradykinin. Notably, in both oxytocin and bradykinin models, GPCR-mediated intracellular calcium response was so minimal with benzbromarone pretreatment that peak fluorescence did not differ cells receiving injections of vehicle instead of GPCR-agonist. Tannic acid (100 μM) pretreatment also significantly reduced oxytocin mediated intracellular calcium elevations to a mean peak fluorescence of 0.64 ± 0.13 (N=10, $p < 0.001$). As expected, pretreatment with NPPB (50 μM) significantly reduced peak fluorescence to 0.47 ± 0.18 (N=10, $p<0.001$) in response to oxytocin and 0.42 ± 0.03 (N=5, $p<0.001$) in response to bradykinin. Benzbromarone produced a greater reduction in peak fluorescence than NPPB (positive control) in response to bradykinin (N=5, $p < 0.001$).

Comment

In this study we show that ANO 1 and 2 are expressed in human and murine USM cells and contribute to pro-contractile electrophysiological currents (STICs) in isolated murine USM cells. We also show that ANO 1 and 2 blockade inhibits spontaneous uterine contractions and inhibits GPCR agonist mediated elevations in intracellular calcium in mice. These findings suggest that ANO 1 and 2 are potentially powerful targets for novel tocolytic agents in uterine smooth muscle cells.

ANO 1 and 2 are expressed at the mRNA level in human and murine myometrium, demonstrating interspecies conservation and underscoring the fundamental importance of ANO 1 and 2 in mammalian USM physiology. Our in situ findings reveal that while ANO 1 and 2 are present on the myometrium, they appear to be absent from uterine vasculature (Fig 1B abcd). While other studies have demonstrated the presence of ANO 1 in pulmonary [21] and cerebral [22] arteries, our immunohistochemical findings suggest a lack of expression in uterine vasculature. Given that commercial antibodies do not distinguish between the isoforms of ANO1 and 2, we are unable to make conclusions about differential distribution. However, our PCR findings do suggest that both ANO 1 and 2 are present in the mammalian myometrium. We confirmed the presence of ANO 1 and 2 persists in cultured cell preparations by immunohistochemical staining, validating the use of cultured cell preparations for study of ANO 1 and 2 (Fig. 1B efgh).

Prior work in our laboratory has demonstrated that ANO 1 and/or 2 are responsible for STICs in airway smooth muscle [16], and others have shown that ANO blockade halts spontaneous transient depolarizations in the interstitial cells of cajal in the GI tract [12]. It

has also been reported that calcium sparks activate both STICs and STOCs (spontaneous transient outward currents) in vascular, gastric, ureteral, vesicular, and airway smooth muscle [23, 24].

In this paper, we demonstrate that STICs are present in myometrial cells and exhibit classic characteristics of CaCCs [9, 10]: calcium activation, voltage dependence, and chloride conductance. We demonstrate calcium activation via GPCR and ryanodine receptor mediated intracellular calcium elevations to activate STICs, and also observed that calcium chelation diminishes STIC amplitude and frequency (data not shown). STICs also exhibit voltage dependence, similar to STICs observed in human airway smooth muscle cells [25, 16] and xenopus oocytes [26]. Using the Nernst equation, the predicted chloride equilibrium potential was −13.8 mV and the measured equilibrium potential under the buffer conditions employed was nearly identical at −13.0 mV, indicating that chloride conductance is responsible for STICs (figure 2A). Finally, STICs are attenuated by pharmacologic blockade of ANO 1 and 2. These findings offer conclusive evidence that myometrial STICs occur as a result of ANO 1 and 2 function, and corroborate historical experiments in which chloride replacement in buffer reduces rat myometrial contractility [33].

In the process of studying murine uterine STICs, we observed qualitative differences in spontaneous currents suggesting two distinct populations of murine USM cells, one displaying consistent STICs and the other displaying cyclic bursts of STICs. The existence of USM cell subpopulations characterized by differing electrophysiological currents has been observed before in rat [27] and human [28] myometrium. Initial work done by Sanders et al on interstitial cells of cajal (ICC) in the GI tract established the seminal functional role of ANO 1 and 2 in allowing for gut pacing. A recently discovered myometrial cell, the telocyte or m-ICLC (myometrial interstitial cajal like cell), may serve a similar function in the myometrium [29]. In fact, blockade of myometrial c-Kit, a marker used to identify gut ICCs, with imatinib halts human uterine contractions [30]. It is possible that ANO 1 and/or 2 may serve an analogous function in this subpopulation of USM cells to their role gut ICCs – as a vital component in establishing rhythmic patterns of contractility? Further study is required, but our data corroborates other investigators in that a USM cell subpopulation exists in the uterus, and the conservation of this finding across species (rat, human, murine) may indicate an important physiologic role for these cells. While these cells did exhibit different electrophysiologic properties, we included them in our analysis because our purpose was to delineate the effect of ANO blockade on all myometrial cells. Interestingly, we found the response to ANO 1/2 inhibition was identical among all uterine smooth muscle cells recorded.

We performed organ bath experiments to further characterize the functional physiologic role of ANO 1 and 2 in uterine contractions. Both the ubiquitous chloride channel blocker niflumic acid and the specific ANO 1/2 antagonist tannic acid significantly reduced both the frequency and amplitude of spontaneous murine uterine contractions. Notably, tannic acid demonstrated enhanced potency compared to niflumic acid in reducing frequency of contraction at an equivalent dose (100uM). This is a novel finding, as previous work in the rat uterus only demonstrated non-specific chloride channel blockade with niflumic acid

alone [27]. The relaxation of spontaneous contractions with an ANO 1/2 specific antagonist in the organ bath suggests that ANO 1 and 2 promote uterine contractility.

To conceptually test the hypothesis that ANO 1 and 2 are participating in GPCR-mediated depolarization that allows for the opening of voltage gated calcium channels, we performed calcium flux studies on monolayers of murine USM cells. Specific ANO 1 and 2 antagonists effects were compared with negative control (vehicle) and positive historical control (ubiquitous chloride channel blockers). All chloride channel inhibitors, ubiquitous and specific, produced significant reductions in GPCR-agonist induced elevations in intracellular calcium, suggesting that ANO 1 and 2 play a vital role in mobilizing calcium to the intracellular cytosolic compartment. The specific CaCC antagonists tannic acid and benzbromarone produced significantly larger reductions in calcium response, indicating that their effect is more potent and specific than ubiquitous blockade.

Our finding suggests the possibility that ANO 1/2 is functionally important for elevations in intracellular calcium flux through cell membrane calcium channels and likely via intracellular release from the sarcoplasmic reticulum (SR). The dependence of SR calcium flux on chloride has been previously proposed [32], and our results necessitate further inquiry, but are suggestive of a pivotal role of ANO 1 and/or 2 in cellular calcium dynamics and by extension USM contractility. Future studies are required to examine if ANO blockade also interferes with Ca^{2+} dynamics and action potential generation in intact tissue.

There are limitations to this study. First, we were not able to identify the differential expression of ANO 1 versus ANO 2 in the myometrium given the difficulty in isolating the two with commercially obtained antibodies. Future studies will address this issue by ANO 1 or 2 knock down to elucidate the differential distribution and functions of these two channels. Second, our functional experiments rely on pharmacologic antagonists, and therefore our results are subject to the pharmacologic sensitivities of the agents used. The study of CaCCs has historically been hampered by a lack of specific pharmacologic antagonists, due in large part to difficulty in the molecular identification of the protein channel responsible for calcium-activated chloride currents. As such, a mainstay for antagonism of these currents historically involved ubiquitous chloride channel inhibitors like niflumic acid and NPPB [27]. However, following the identification of ANO 1 and 2 in 2008, specific inhibitors for CaCCs were developed. Tannic acid, a member of the gallotannin family, was discovered by Namkung et al in 2012 to be a potent inhibitor of CaCCs but not the CFTR chloride channel [18]. Benzbromarone was identified by Huang et al in 2012 as part of a high throughput screen for CaCC inhibitors and was found to be both potent and specific to the CaCC channel without exerting effects on CFTR or IBMX chloride channels in NHBE cells [17]. We chose these selective CaCC antagonists in all studies and used ubiquitous chloride channel blockers with known historical efficacy for inhibiting calcium activated chloride currents as positive controls. While it is possible that off-target pharmacologic effects might contribute to some of the effects we observed (ex. inhibition of calcium responses) – we purposely employed multiple drugs (from different drug classes) known to antagonize ANO 1 and 2 channels to minimize this possibility. Future studies utilizing ANO gene knockdown or silencing are required to corroborate our findings.

Finally, we used non-gravid animals, and further studies must be performed on pregnant myometrium to substantiate CaCCs as potential tocolytic agents.

In summary, we have shown that the calcium activated chloride channels ANO 1 and 2 are expressed in human and murine USM cells and that these channels are intimately involved in the normal physiology of murine uterine contractility. These findings introduce ANO 1 and 2 as novel channels involved in the normal physiology of uterine contractions. We have shown that blockade of ANO 1 and 2 has a powerful tocolytic effect in the organ bath, suggesting that in vivo targeting of ANO 1 and 2 may arrest the progression of spontaneous preterm labor.

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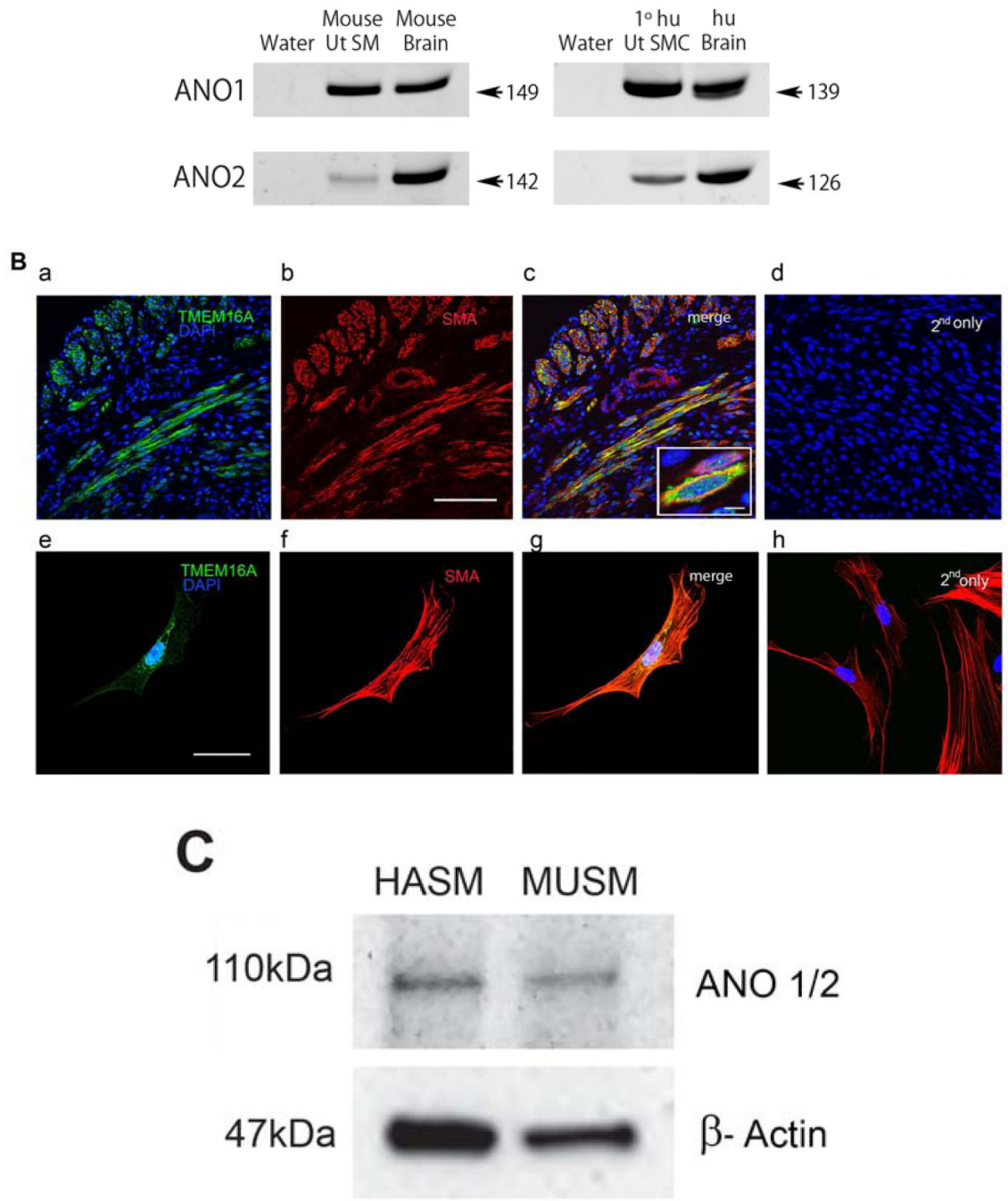

Figure 1A. RT-PCR of ANO 1 and 2 in murine and human USM

RT-PCR data showing qualitative evidence of ANO 1 and 2 expression in both human and murine uterine smooth muscle, compared against negative control (water) and positive control (brain). Control bands are present at expected sizes. (Ut SM = intact murine uterine smooth muscle, 1 hu Ut SMC: primary human uterine smooth muscle cultured cells).

B. Immunohistochemical detection of ANO 1 and 2 on murine myometrium and cultured USM cells.

a-c. Immunofluorescence labeling of intact murine myometrium using antibodies against TMEM16A = ANO 1/2 (green), DAPl nucleus stain (blue) and F-actin (rhodamine phalloidin, red).**c**. Merged image with inset illustrating co-expression **d**. Negative control with incubation of the secondary reporter antibodies and DAPI only. Calibration bar represents 35 μm.

e-g. Double labeling of ANO 1 (green) and F-actin (rhodamine phalloidin, red) on cultured cells. **h**. Negative control with incubation of the secondary antibody, DAPI and F-actin. Calibration bar represents 10 μm.

C. Immunoblotting for ANO 1 and 2 protein expression on cultured murine USM cells. Representative Immunoblot demonstrating immunoreactive bands of expected molecular mass (~110kDa) for the ANO 1/2 protein is expressed in mouse uterine smooth muscle primary cultured cells (MUSM). Cultured cells of human airway smooth muscle (HUSM) is included as a positive control. Presence of β-Actin was used to illustrate comparable sample loading.

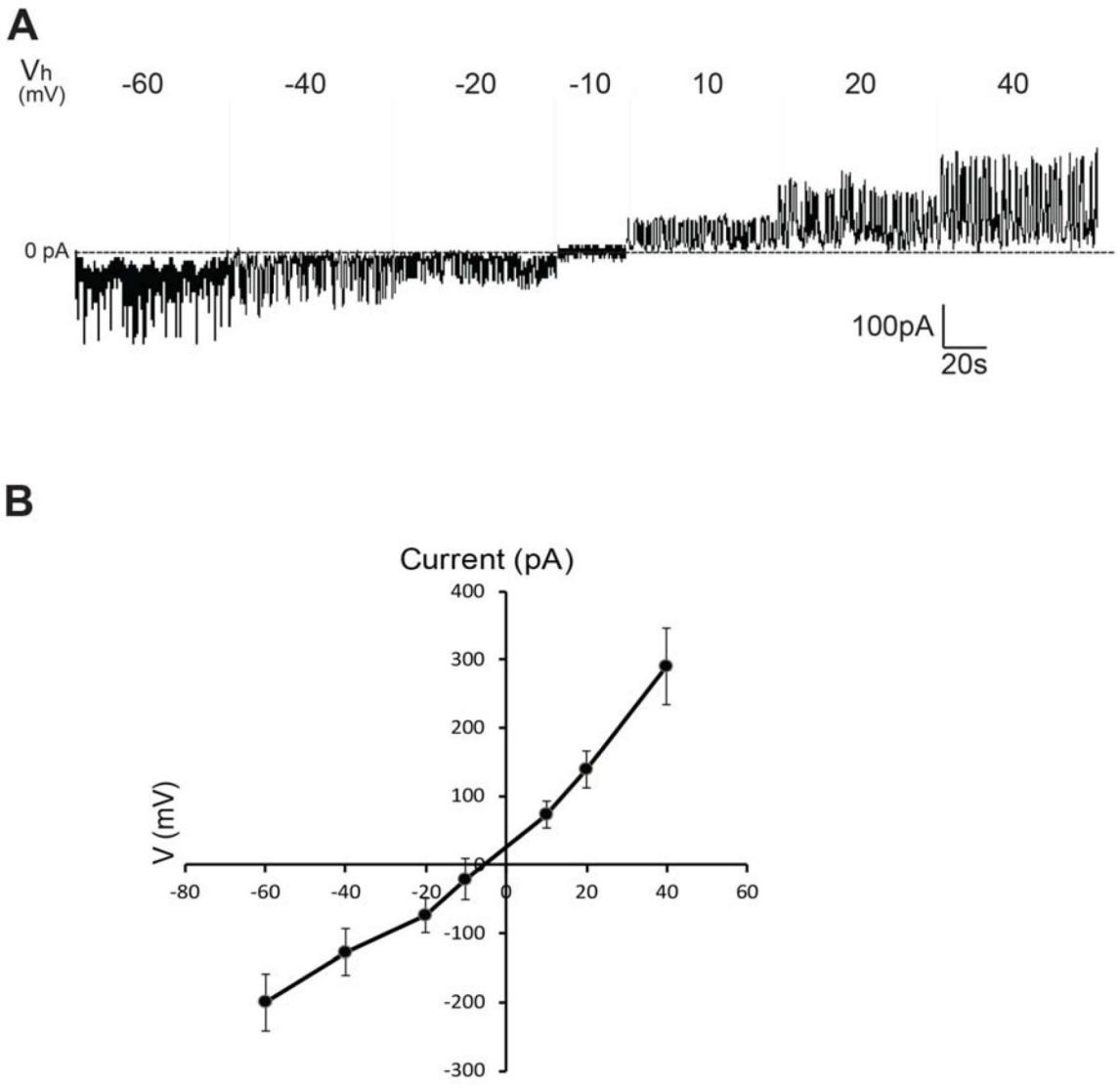

Figure 2. Current–voltage (I/V) relationship of STICs in murine USM cells

A. Representative tracing of STICs recorded at different holding potentials (Vh).

B. Relationship between mean peak amplitude of STICs (n=5) and voltage holding potential. The reversal potential for STICs is −12.7 ± 1.2 mV (n=5).

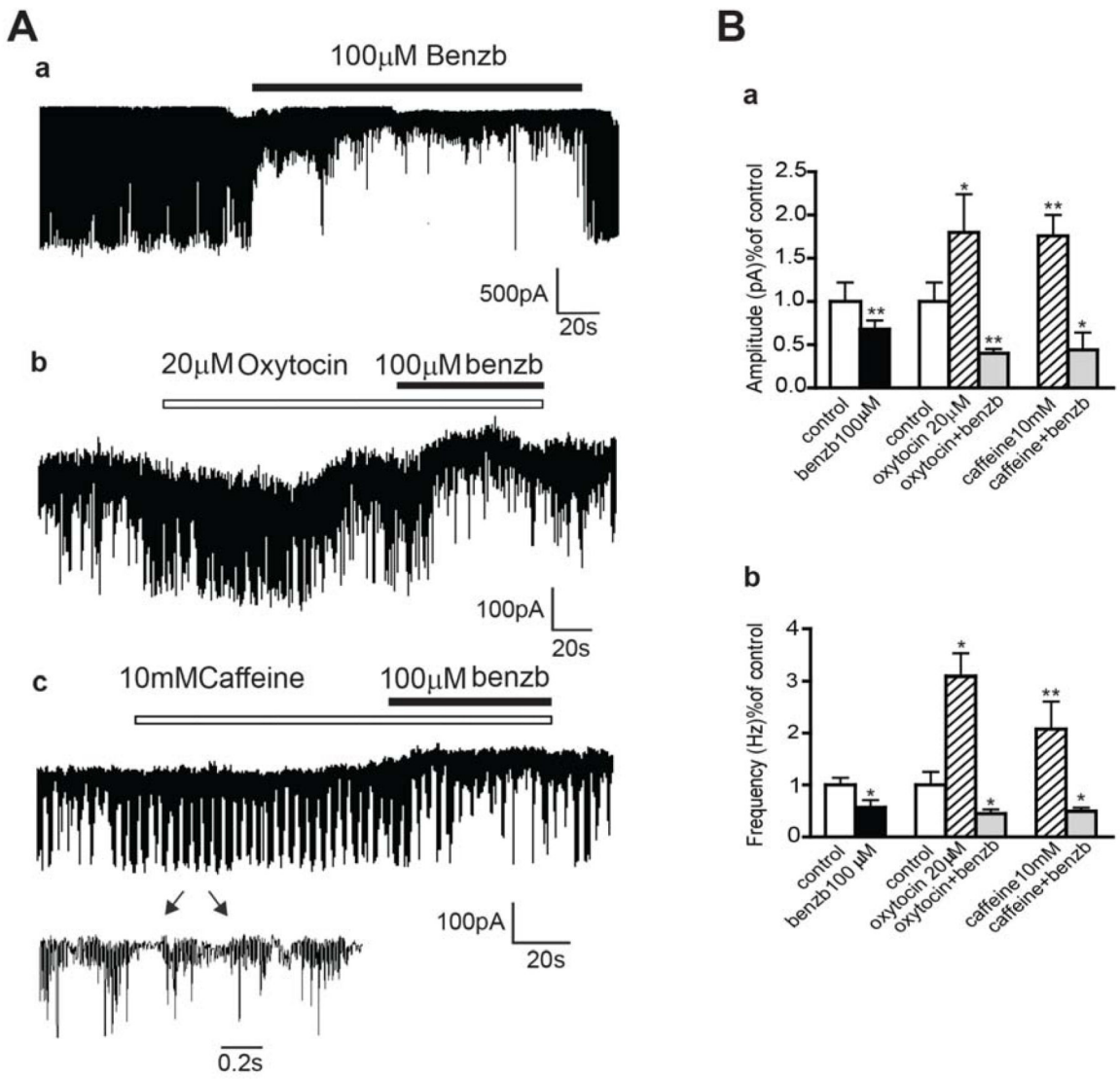

Figure 3. Effects of benzbromarone, oxytocin and caffeine on STICs in murine USM cells

A Representative whole cell recordings of STICs: a. Current recorded in response 100 μM benzbromarone (filled bar, n=8). **b**. current recorded in response to both 20μM oxytocin (open bar) and 100 μM benzbromarone (filled bar, n=5). **c**. 10 mM caffeine (open bar) and 100 μM benzbromarone (filled bar, n=5). Holding potential is −60 mV. **Inset**. Representative tracing of cells displaying auto-rhythmicity.

B a-b. Summary of the effects of benzbromarone, oxytocin and caffeine on amplitude (**a**) and frequency (**b**) of STICs. The second blank bar represents the control group, used for paired comparisons to oxytocin and caffeine treatments (n=10). Oxytocin and Caffeine treatment enhanced STIC amplitude and frequency. ANO 1/2 blockade with benzbromarone (benzb) suppressed both basal STICs as well as oxytocin and caffeine induced enhancement of STICs (*p <0.05; **p <0.01).

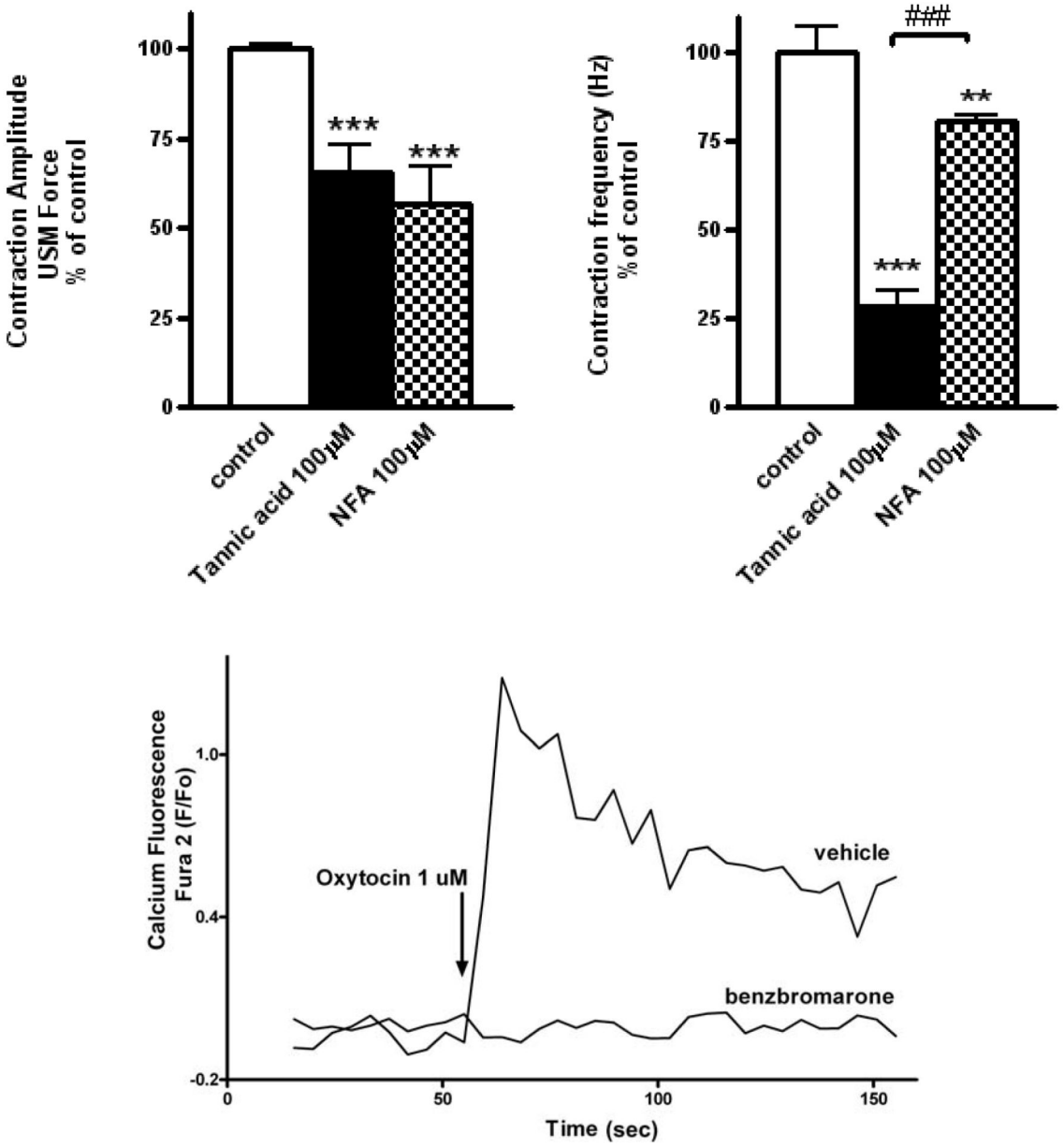

Figure 4. ANO 1/2 antagonism and spontaneous murine USM contractility

A. Representative force tracings show inhibition of spontaneous murine USM contractions following treatment with tannic acid 100 μM (selective ANO antagonist; bottom tracing - Blue) vs.vehicle control (upper tracing - Red).

B. **Compiled Contractility Assessments: Left graph**. Shows amplitude of muscle contraction in grams compared to control. Both tannic acid and niflumic acid differed significantly from vehicle control, but not from each other (*** $p < 0.001$).

Right graph. Shows contraction frequency compared to control. Both tannic and niflumic acid reduced the frequency of contractions (*** $p < 0.001$ and ** $p < 0.01$ respectively). Tannic acid reduced frequency significantly more than did niflumic acid ($^{###}$ $p < 0.001$).

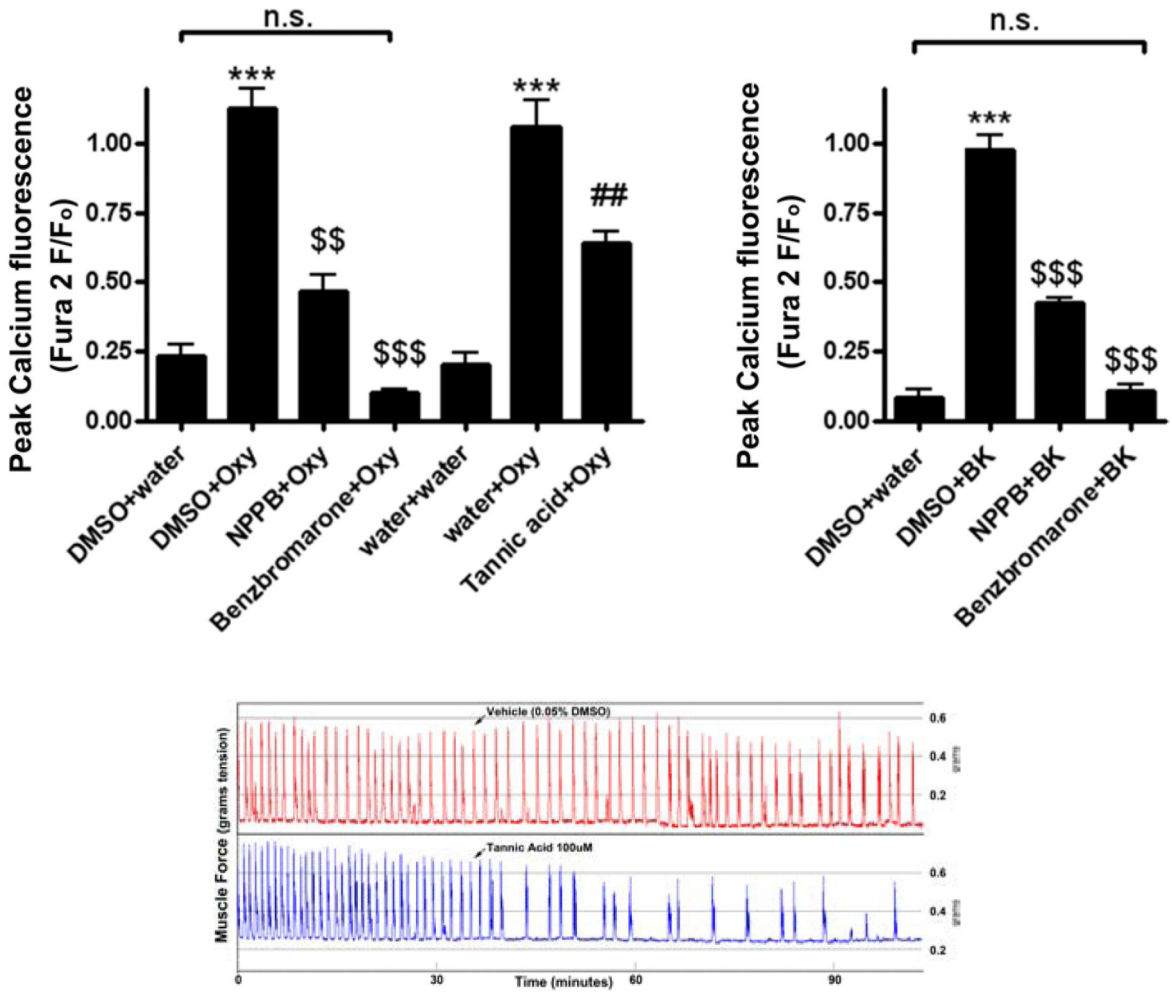

Figure 5. Peak intracellular calcium in response to Gq coupled agonist after pretreatment with ANO 1/2 selective and ubiquitous chloride channel antagonists

A. Representative tracing of Fura-2 fluorescence versus time, with 1μM oxytocin added at 55s. In cells pretreated for 10 minutes with 50μM benzbromarone, oxytocin mediated increase in intracellular calcium is suppressed.

B. Compiled results on evoked intracellular calcium responses (peak fluorescence) in murine uterine smooth muscle cells. All experiments were compared to their vehicle controls (0.1% DMSO for NPPB and benzbromarone, HBSS for oxytocin and tannic acid). **Left Graph**. 1μM oxytocin administration significantly elevated calcium levels compared to vehicle administration (*** $p<0.001$). Oxytocin-mediated elevations in intracellular calcium fluorescence was significantly attenuated in cells receiving 10 minute pretreatments with 50μM NPPB, 50μM benzbromarone, or 100μM tannic acid, compared with DMSO pretreatment control ($$ $p<0.01$, $$$ $p<0.001$, ## $p<0.01$). “n.s.” represents a lack of significant difference between vehicle control and benzbromarone pretreatment (n=6). **Right Graph**. 10μM bradykinin administration also significantly elevated peak calcium levels compared to vehicle administration (*** $p<0.001$). Bradykinin-mediated elevations in intracellular calcium responses are significantly attenuated in cells receiving 10 minute

pretreatments with 50μM NPPB or 50μM benzbromarone. “n.s.” represents a lack of significant difference between vehicle control and benzbromarone pretreatment (n=5).

Table 1
Human/murine ANO 1 and 2 oligonucleotide primer pairs

Species	Gene	Genebank Accession Number	Amplicon Size	Sequence (5′ → 3′)
Human	ANO1	NM_018043	139bp	GGAAGCGGAAACAGATGC GACTCAACTA
				TTTCTGGACTCTTTCTTCA GAGACTTCTCCAA
	ANO2	NM_001278596	126bp	CCCGAGTTCCAGAACACA GCAACAACAA
				TGCGGCTGCGGGTGGCAT TATCAAA
	GAPDH	NM_002046	213bp	CCAGGGCTGCTTTTAACTC TGGTAAAGTGGATA
				CATCGCCCCACTTGATTTTGGAGGGA
Mouse	ANO1	NM_178642	149bp	CTCTGGCTCCACTCTTCGCCCTGCTAAA
				CAGCTTCCCAACACCTCTGAGGATGTTA
	ANO2	NM_153589	142bp	CGTGGTCATCCTCATCTTGGATGAGATCTAT
				GGAGTAGGCATTGACAAACTTGAGCAAGAA
	GAPDH	NM_008084	156bp	GACAAAATGGTGAAGGTCGGTGTGAA
				AGTGGAGTCATACTGGAACATGTAGACCATGTAG