University of Nevada, Reno

Generation of Muscle Tone in the Murine Internal Anal Sphincter by Anoctamin 1

A thesis submitted in partial fulfillment of the requirements for the degree of

BACHELOR OF SCIENCE, BIOCHEMISTRY AND MOLECULAR BIOLOGY

by

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May, 2015

UNIVERSITY OF NEVADA THE HONORS PROGRAM RENO

We recommend that the thesis prepared under our supervision by

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entitled

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ABSTRACT

Maintenance of normal muscle tone in the internal anal sphincter is important to prevent medical disorders related to incontinence. Recently, it has been found that a calcium activated chloride channel found in interstitial cells of cajal, anoctamin 1, has a role in the generation of slow waves in smooth muscle cells. In this study, the role of anoctamin 1 in the generation of muscle tone in the internal anal sphincter was further investigated. Electrophysiology measurements confirmed that slow waves were entirely abolished when the murine internal anal sphincter was exposed to T16Ainh-A01, a known inhibitor of anoctamin 1. According to strain gauge measurements, muscle tone decreased in a dose-dependent manner in the presence of T16Ainh-A01. Furthermore, inhibiting L-type calcium channels in smooth muscle with nifedipine also resulted in dose dependent smooth muscle relaxation. This data leads to the conclusion that muscle tone in the murine internal anal sphincter results from depolarization of the interstitial cells of cajal by the efflux of chloride through anoctamin 1, and propogation of this signal within the smooth muscle via L-Type calcium channels.

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INTRODUCTION

Malfunctions in the proper maintenance of muscle tone in the internal anal sphincter (IAS) can cause several medical problems; while it is important that the IAS relax when necessary, absence of normal muscle tone can lead to fecal incontinence. The IAS has unique properties when compared to other tissues, most notably increased muscle tone and faster slow wave pace as compared to the rectum or colon (Mutafova-Yambolieva et al. 2003). However, the mechanism of tone generation in the IAS is still uncertain. One protein that seems to be particularly important to this mechanism is anoctamin 1 (Ano1, MGI:2142149). Ano1 was recently grouped into a specific class of proteins called calcium activated chloride channels (CaCC's) based upon decreased anion transport when Ano1 is inhibited or cytosolic calcium is removed (Caputo et al. 2008, Yang et al. 2008, Shroeder et al. 2008, Manoury 2010, Scudieri et al. 2011). These CaCC's are transmembrane proteins that allow for an efflux of chloride in the presence of cytosolic calcium, depolarizing the cell and causing smooth muscles to contract (Large & Wang 1996).

Recently, studies have found that Ano1 in particular has a role in slow wave generation. Inhibition of Ano1 by niflumic acid in the murine gastric antrum and small intestine has resulted in a decrease in the magnitude of slow waves in a concentration dependent manner (Hwang et al. 2009). It is thought that the summation of asynchronous slow wave activity across neighboring smooth muscle cells produces muscle tone in the IAS (Hall et al. 2014), further

supporting the hypothesis that Ano1 is integral for tone generation. Furthermore, these results have wider implications about the role of interstitial cells of cajal (ICC's) in slow wave propagation, as the *ANO1* gene, also referred to as *TMEM16a,* is expressed at higher levels in ICC's than any other cell type (Huang et al. 2009, Gomez-Pinilla et al. 2009). However, while ICC networks are still maintained in the small intestine of *TMEM16a-/-* mice, slow waves are entirely abolished, implying that the Ano1 protein is imperative in the function of ICC's to generate slow waves and, therefore, contribute to cell contraction (Hwang 2009).

In the past, niflumic acid has been used as the most common inhibitor for Ano1 and CaCC's. Although niflumic acid is a potent inhibitor of CaCC's (Large & Wang 1996), it may affect other channels or proteins as well. Other chloride channels, like the cystic fibrosis transmembrane conductance regulator, have been shown to be affected by many commonly used CaCC inhibitors (Scudieri et al. 2011). Furthermore, a recent study found that niflumic acid causes the release of calcium from internal cellular storage, which might be responsible for the changes in tissue behavior that were attributed to Ano1 (Cruickshank et al. 2003). In order to support the conclusion that Ano1 is responsible for observed changes based upon its role as a CaCC, it is necessary to use a specific inhibitor that does not affect calcium metabolism or other chloride channels. For the purposes of this study, T16Ainh-A01 will be used to observe the electrical and mechanical effects of Ano1 inhibition, as it has been shown to inhibit Ano1

currents in a dose dependent manner (Bradley et al. 2014). Also, this inhibitor does not affect L-type calcium channels (Sung et al. unpublished). Based upon past experiments, inhibition of Ano1 with this specific inhibitor is expected to block slow waves in smooth muscle cells of the murine IAS and inhibit muscle tone in a dose dependent manner.

The method by which ICC depolarization might initiate contraction within the neighboring smooth muscle cells is still unclear. However, exploration of the role of L-type calcium channels (Ca_V_L's) might provide further insight into this question. Ca_V_L's are voltage gated channels that produce a long-lasting inward current of calcium necessary for smooth muscle contraction (Reuter 1985). In order to further investigate this relationship, Ca_V_L's will here be inhibited by nifedipine. All 1,4-dihyropyridines inhibit Ca_V_L's without affecting other calcium channels (Hess et al 1985), and, of these types of drugs, nifedipine has become a standard inhibitor for Ca_V_L's. In this study, nifedipine will be used to inhibit Ca_V_L's. Based upon the clear role of Ca_V_L's in smooth muscle contraction, it's thought that nifedipine will inhibit muscle tone in a dose dependent manner.

METHODS

Tissue Preparation

Mice used for these studies were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experiments and procedures were approved by the Institutional Animal Use and Care Committee at the University of Nevada. C57BL/6 (30–90 days old;

Jackson Laboratory, Bar Harbor, MN, USA) were killed with isoflurane (Baxter, Deerfield, IL, USA) followed by cervical dislocation. Rectoanal segments from each species were mounted in a dissection dish containing cold Krebs bicarbonate solution (KRB) of the following composition (in mM): NaCl 118.5, KCl 4.7, CaCl2 2.5, MgCl2 1.2, NaHCO3 23.8, KH2PO4 1.2, dextrose 11.0. This solution had a pH of 7.4 at 37°C when bubbled to equilibrium with 95% O2–5% CO2. The rectoanal region was cleared of all adhering skeletal muscle, adipose tissue, and glands, opened longitudinally, and the mucosa removed. 2 mm wide muscle stips adjacent to the distal most extension of the IAS were created.

Electrophysiological Measurements

Muscle strips were pinned to the base of an electrophysiologic chamber with the submucosal surface facing upwards. Smooth muscle cells were impaled with glass microelectrodes (filled with 3 M KCl; tip resistances ranging from 60 to 150 MΩ). Nerves were stimulated via platinum electrodes (1 cm length) placed 1 mm away from the muscle at both sides along its entire length. 20 μM wortmannin was introduced to the electrophysiological chamber for 15 minutes, followed by a 30 minute washout period, in order to block contraction and maintain tissue impalements while allowing electrical activity to recover to untreated levels (Burke et al. 1996). Impalements were characterized by a drop in membrane potential to -40 mV and a return in membrane potential to zero upon removing the electrode from the cell. All experiments were performed in the

presence of 1 μM atropine and 1 μM MRS2500 in order to eliminate the possible contribution of cholinergic nerves and purinergic relaxation.

Contractile Experiments

Muscle strips were attached with sutures to a stable mount and to a Gould strain gauge and immersed in tissue baths containing 15 mL of oxygenated KRB solution maintained at 37 C. A basal tension of 0.25 g was applied to mouse muscle strips. Tissues were equilibrated during the first 45 minutes after mounting to allow active tone and phasic contractile activity to develop. Data were stored and analyzed by a computer using a data acquisition program (AcqKnowledge; Biopac systems Inc., Goleta, CA, USA). All experiments were performed in the presence of 1 μM atropine, 1 μM MRS2500, and 100 μM L-NNA in order to eliminate the possible contribution of cholinergic nerves, purinergic relaxation, and nitrergic relaxation. Maximum relaxation was determined by the addition of the nitric oxide donor, sodium nitroprusside (SNP, 10 μM).

Solutions and Drugs

Atropine sulphate, N(ω)-nitro-L-arginine (L-NNA), sodium nitroprusside (SNP), and nifedipine were purchased from Sigma (Saint Louis, MO, USA). Wortmannin was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). MRS2500 was purchased from Tocris Bioscience (Ellisville, MO, USA). T16Ainh-A01 was purchased from EMD Millipore Corporation (Billerica, MA,

USA). Wortmannin and T16Ainh-A01 were dissolved in DMSO. All other drugs were dissolved in deionized water.

RESULTS

Slow wave activity in presence of T16Ainh-A01

Ten μM T16Ainh-A01 was introduced into the electrophysiology chamber through a passive drip system after normal slow wave activity had been recorded. In six murine IAS's studied, the average resting membrane potential before introduction of the inhibitor was -49.0±1.4 mV and the average slow wave amplitude was 22.3±8.0 mV. Introduction of 10 μM T16Ainh-A01 resulted in an average resting membrane potential of -55.0±3.1 mV and an average slow wave amplitude of 1.6±0.6 mV. Slow wave amplitude significantly decreased upon inhibition of Ano1 (paired $t(5) = 6.1804$, $p = 0.0016$). Furthermore, the cell hyperpolarized significantly following slow wave abolition (paired $t(5) = 3.2049$, $p = 0.0239$), with a change in membrane potential of -6.0±4.6 mV. The change in membrane potential ranged from -1.8 mV to -14 mV, and slow wave amplitude was abolished regardless of cell hyperpolarization. A summary of the effect of Ano1 inhibition on slow wave amplitude and membrane potential is provided in Figure 1.

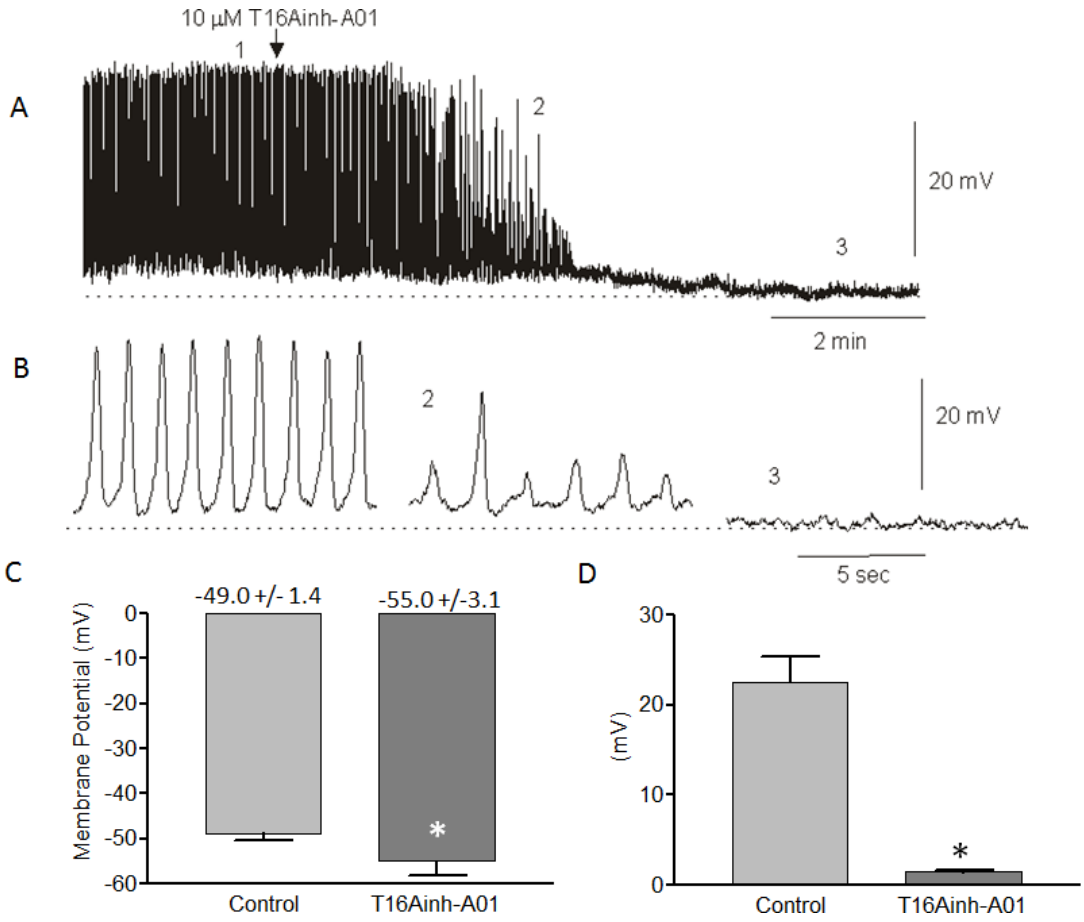

Figure 1. Effect of Ano1 inhibition on slow wave amplitude and membrane potential in murine smooth muscle cells of the IAS. A shows the large scale effect of Ano1 inhibition on slow wave amplitude and hyperpolarization. Ten µM T16Ainh-A01 was introduced into the drip system at the location marked, allowing it to reach the bath ~2 minutes later. B shows the smaller scale slow wave amplitude before and after the introduction of the Ano1 inhibitor, including an intermediate change as the inhibitor began to take effect. C shows the average membrane potential across six tissues before and after introduction of the inhibitor; Ano1 inhibition resulted in a significant hyperpolarization. D shows the average slow wave amplitude across six tissues before and after the introduction of the inhibitor; Ano1 inhibition resulted in a significant decrease in slow wave amplitude.

Contractile activity in presence of T16Ainh-A01 or nifedipine

Once normal contraction had been recorded, each drug was introduced into the tissue bath at increasing concentrations. Several minutes were allotted between each increase in concentration to allow the tissue to respond to the drug. Seven tissues were tested on the following concentrations of T16Ainh-A01: 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, and 20 μM. There was a dose dependent relationship between the concentration of T16Ainh-A01 and muscle relaxation. The largest relaxation occurred between 3 μM and 10 μM T16Ainh-A01 (paired $t(6) = 6.3978$, $p = 0.0007$). Six tissues were tested on the following concentrations of nifedipine: 0.1 nM, 0.3 nM, 1 nM, 3 nM, 10 nM, 30 nM, and 100 nM. There was a dose dependent relationship between the concentration of T16Ainh-A01 and muscle relaxation. The largest relaxation occurred between 10 nM and 30 nM nifedipine (paired $t(5) = 5.6333$, $p = 0.0024$).

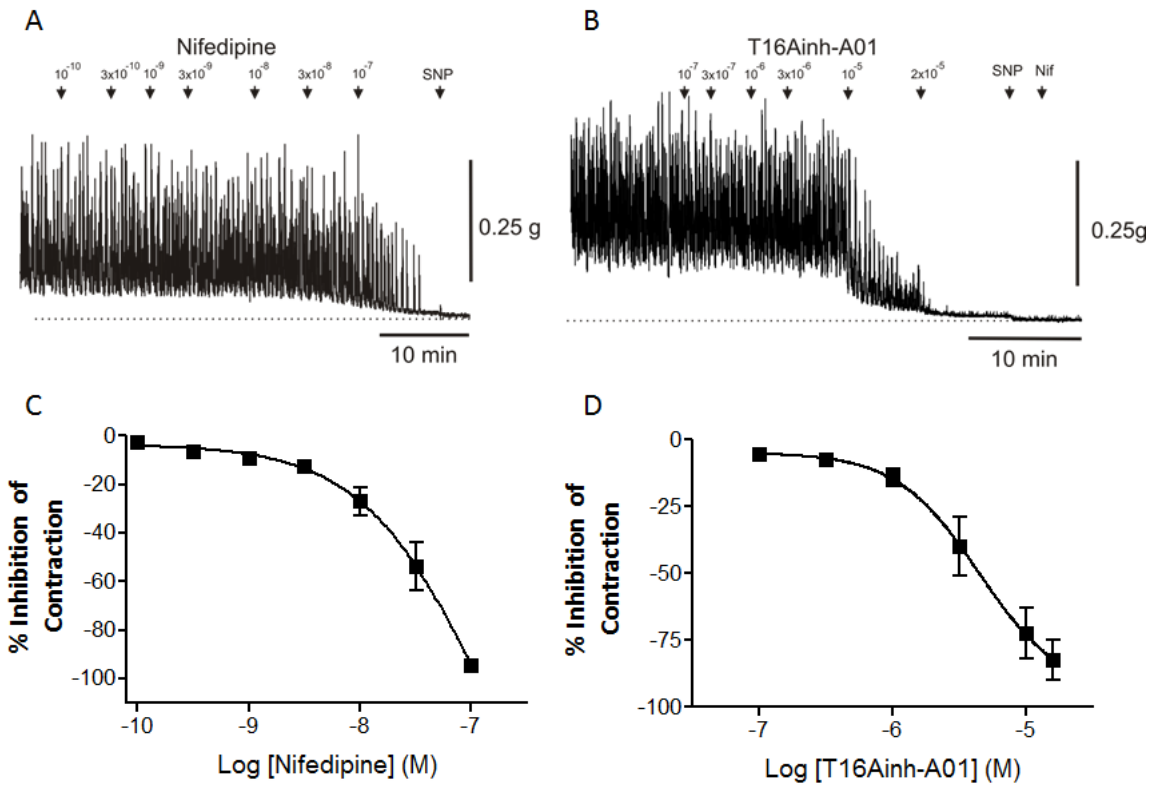

Figure 2. Dose dependent effect of CaCC blockers on contractile activity of the murine IAS. A shows the effect of the indicated molarity of nifedipine on muscle contraction. B shows the effect the indicated molarity of T16Ainh-A01 on muscle contraction. The average grams of contractile force following each increase in drug concentration were used to calculate the relaxation of the muscle. C shows the dose dependent relationship between relaxation and nifedipine concentration. D shows the dose dependent relationship between relaxation and T16Ainh-A01 concentration.

DISCUSSION

In this study, Ano1 was inhibited in order to understand the importance of this protein in slow wave generation and muscle contraction in smooth muscle of the IAS. As shown in Figure 1, introduction of TMEM16Ainh-A01, a specific inhibitor of the Ano1 protein, abolished slow waves in smooth muscle cells. Smooth muscle cells also hyperpolarized following inhibition of Ano1, which is to be expected considering Ano1's role in normal depolarization through efflux of chloride; without this efflux of chloride, the cell will hyperpolarize. However, it is important to note that the hyperpolarization of the cell occurred after the slow waves had been abolished, indicating that the slow waves were affected directly by the inhibition of the Ano1 protein and not by the hyperpolarization of the cell. Furthermore, TMEM16Ainh-A01 relaxed muscle tone in the IAS in a dose dependent manner, as shown in Figure 2. This supports the conclusion that Ano1 is vital for maintenance of normal muscle tone in the IAS. Inhibition of Cav_L's through introduction of nifedipine also relaxed muscle tone in a dose dependent manner, indicating that tone results from an increase in CavL activity in smooth muscle. This activity most likely occurs in response to depolarization via slow waves initiated by ANO1 in ICC's.

Considering that Ano1 is expressed and localized more highly in ICC's than any other cell type in the IAS, the vital role of Ano1 in slow wave propagation and muscle contraction has large implications for the role of ICC's in these processes. ICC's have begun to draw attention in the field of

gastroenterology, as motility disorders such as chronic constipation are associated with ICC injury (Huizinga & Chen 2014). In recent years, ICC's have been tested for their role in slow wave propagation using W/Wv knockout mice, which knock out the c-kit protein involved in ICC's expression. Immunohistological studies have not detected c-kit in the IAS of W/Wv knockout mice, although slow waves persisted (Duffy et al. 2012, Cobine et al. 2011). While many have taken this to suggest that ICC's are not solely responsible for slow wave generation in smooth muscle cells, it is thought that loss of the c-kit receptor might not cause loss of ICC cells, although it would cause a loss in c-kit immunostaining (Betolli et al. 2012, Cloud 1993). Furthermore, while W/Wv mice stained with c-kit had 50-60% of normal c-kit density, W/Wv mice still have a normal distribution of Ano1 positive cells, indicating that Ano1 might be a more effective immunostaining candidate for ICC's (Wang et al. 2014). Based on the evidence that Ano1 is expressed highly in ICC's, the results of this study provide further evidence that ICC's play an important role of slow wave generation and propagation.

However, based upon recent studies that have shown Ano1 to be expressed within vascular smooth muscle cells (Davis et al. 2010, Manoury 2010, Namkung et al. 2010, Schreiber et al. 2010), some are claiming that the integral role of Ano1 in the IAS is through smooth muscle cells, not ICC's. In order to further investigate this hypothesis, the extent of ICC loss within W/Wv mice must be further explored, to see whether this knockout provides a useful

model for ICC analysis. Immunostaining of the Ano1 protein in conjunction with different cell types within the IAS should resolve the cellular location of this protein. Furthermore, comparison of these immunohistochemical images between wild type and W/Wv mice might allow the loss of ICC cells within W/Wv mice to be more concretely distinguished.

Because this study helps to characterize Ano1, it may also provide insights into the roles of this protein outside of the IAS. Ano1 is expressed widely in most murine tissues and to a lesser extent in human tissues (Schreiber et al. 2010). Because Ano1 is expressed in all ICC's, not just those that produce slow waves, Stanich et al. chose to study some of the other roles Ano1 might have within ICC's in 2011. They found that Ano1 also signals ICC proliferation through the efflux of chloride (Stanich et al. 2011). This is further related to several articles that have linked over-expression of Ano1 to cancer (West et al. 2004, Carneiro et al. 2008).

It's possible that these different roles are indicated by alternative splicing of the Ano1 protein. It has been shown that the *ANO1* gene participates in alternative splicing, particularly in those exons that cross the membrane (Caputo et al. 2008). Differences in alternative splicing might contribute to the differences seen in slow wave activity across tissue types. For instance, slow waves occur at a smaller amplitude and frequency in the rectum than in the IAS (Mutafova-Yambolieva et al. 2003). Some of these changes may be attributed to alternative splicing between cell types. Similarly, this sort of alternative splicing

might be an indicator of the different roles of Ano1. This is particular important because alternative splicing is comparable between mice and humans, which allows mice to be used as model organisms (O'Driscoll et al 2011). Further investigation into alternative splicing might provide information on how this important protein functions. Data from the present study can be useful to illustrate the importance of Ano1 within the IAS.

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