Published in final edited form as:

Neurogastroenterol Motil. 2012 March ; 24(3): e125–e135. doi:10.1111/j.1365-2982.2011.01848.x.

Lesioning of TRPV1 Expressing Primary Afferent Neurons Prevents PAR-2 Induced Motility, but Not Mechanical Hypersensitivity in the Rat Colon

Shelby K. Suckow^b, Ethan M. Anderson^b, and Robert M. Caudle^{a,b}

Shelby K. Suckow: shelbyks@gmail.com; Ethan M. Anderson: ethan241@ufl.edu; Robert M. Caudle: caudle@ufl.edu

^aDepartment of Oral and Maxillofacial Surgery, University of Florida College of Dentistry, Gainesville, FL 32610, USA

^bDepartment of Neuroscience, University of Florida College of Medicine, McKnight Brain Institute, Gainesville, FL 32610, USA

Abstract

Background—Proteinase activated receptor 2 (PAR-2) is expressed by many neurons in the colon, including primary afferent neurons that co-express transient receptor potential vanilloid 1 (TRPV1). Activation of PAR-2 receptors was previously found to enhance colonic motility, increase secretion and produce hypersensitivity to mechanical stimuli. This study examined the functional role of TRPV1/PAR-2 expressing neurons that innervate the colon by lesioning TRPV1 bearing neurons with the highly selective and potent TRPV1 agonist resiniferatoxin.

Methods—Colonic motility in response to PAR-2 activation was evaluated in vitro using isolated segments of descending colon and in vivo using manometry. Colonic mechanical nociceptive thresholds were measured using colorectal distension. TRPV1 expressing neurons were selectively lesioned with resiniferatoxin.

Key Results—In vitro the PAR-2 agonists trypsin and SLIGRL did not alter contractions of colon segments when applied alone, however, the agents enhanced acetylcholine stimulated contraction. In vivo, PAR-2 agonists administered intraluminally induced contractions of the colon and produced hypersensitivity to colorectal distension. The PAR-2 agonist enhancement of colonic contraction was eliminated when TRPV1 expressing neurons were lesioned with resiniferatoxin, but the PAR-2 agonist induced hypersensitivity remained in the lesioned animals.

Conclusions and Inferences—Our findings indicate that TRPV1/PAR-2 expressing primary afferent neurons mediate an extrinsic motor reflex pathway in the colon. These data, coupled with our previous studies, also indicate that the recently described colospinal afferent neurons are nociceptive, suggesting that these neurons may be useful targets for the pharmacological control of pain in diseases such as irritable bowel syndrome.

Keywords

colon; colospinal afferent neurons; dorsal root ganglion neurons; proteinase activated receptor 2; transient receptor potential vanilloid 1

Correspondence: Robert M. Caudle, Department of Oral and Maxillofacial Surgery, University of Florida College of Dentistry, PO Box 100416, Gainesville, FL 32610, USA, caudle@ufl.edu, Phone: 1-352-273-6767, Fax: 1-352-392-7609.

Disclosures

The authors declare that they have no competing interests.

Introduction

The role of proteinase activated receptor 2 (PAR-2) in the colon has been studied extensively and it is clear that PAR-2 plays a pivotal part in several bowel disorders. PAR-2 belongs to a class of G-protein coupled receptors that are activated by serine proteases (1). Activation of PAR-2 in the colon induces inflammation (2–6), stimulates enteric neurons (7–9), induces visceral pain (8), stimulates secretion into the lumen (10–14) and alters visceral smooth muscle tension (15;16). Interestingly, PAR-2 is expressed in enteric neurons, visceral primary afferent neurons and secretory epithelial cells in the colon (14;17–19). This expression pattern is consistent with the effects of PAR-2 agonists. Furthermore, the distribution and function of PAR-2 in the colon correlates well with the symptoms of diarrhea predominant irritable bowel syndrome (IBS) (20–26). The fact that IBS patients also have elevated levels of serine proteases in their stools suggests the hypothesis that activation of PAR-2 receptors by these proteases induces the symptoms of the disease (21).

In animal models PAR-2 activation sensitizes the colon to mechanical stimuli, increases the colon's sensitivity to capsaicin and induces referred hypersensitivity in the hind paws (8). PAR-2 is co-expressed on transient receptor potential vanilloid 1 (TRPV1) expressing primary afferent neurons in dorsal root ganglia (DRG) (8;27;28). The co-localization data support the enhancement of capsaicin's, a TRPV1 agonist, responses by PAR-2 agonists (8). In a previous study we demonstrated that lesioning of TRPV1 expressing DRG neurons with the TRPV1 agonist resiniferatoxin eliminated all PAR-2 and TRPV1 expression in the DRGs. The lesions did not significantly influenced the amount of PAR-2 expression in the colon while eliminating all TRPV1 labeling in the colon (28). The most interesting aspect of this study was that the lesions did not influence nociceptive thresholds to colorectal distension. Additionally, we found that these resiniferatoxin lesions did not influence hypersensitivity to colorectal distension in rats with colonic inflammation. Our data suggested that colospinal afferent neurons, which also express PAR-2, may be responsible for nociceptive transmission. However, following the lesions there remained colonic afferent neurons in the DRGs that did not express either TRPV1 or PAR-2 (28;29). It is possible that these residual colonic DRG neurons carry nociceptive information from the sensitized colon. Thus our previous data indicated that there are three separate afferent pathways that may transmit nociceptive information from the colon to the spinal cord. These pathways consist of the TRPV1/PAR-2 expressing DRG colonic afferents, the DRG colonic afferents that lack both TRPV1 and PAR-2 and the colospinal afferent neurons, which express PAR-2 but not TRPV1 (28;29). To distinguish the function of these three pathways we have now examined the effects of PAR-2 agonists on rat colon. Since PAR-2 agonists will only act directly on the TRPV1/PAR-2 expressing DRG neurons and on the colospinal afferent neurons it is possible to separate the function of the three colonic afferent neuron types by using resiniferatoxin to lesion TRPV1 expressing neurons. These data provide new clues as to the function of the PAR-2 expressing neurons in the colon.

Methods

Animals

Experiments were performed on male Sprague-Dawley rats weighing 200–250g. They were housed in pairs with free access to food and water in the University of Florida's animal care facility with a 12-h light/dark cycle. These facilities are AAALAC accredited. All experiments conformed to guidelines on the ethical use of animals as published by the International Association for the Study of Pain. All procedures were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

In vitro gut assay

Descending colon segments (2 cm, 32 segments from 16 rats, with mucosa intact), were removed from naive rats and mounted in individual tissue chambers. The segments were preloaded with two grams of force. Circular muscle contractions were recorded in a bath of Mg^{2+} -free Tyrodes solution (mM; NaCl, 140; KCl, 4; $CaCl_2$, 4; Glucose, 10; pH 7.4). Acetylcholine (ACh) (200 μ M), trypsin (10nM) or the peptide Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL; 100 μ M), and Trypsin or SLIGRL combined with ACh were applied to the tissue for 10 minutes in the Tyrodes solution. Trypsin and SLIGRL were examined in different experiments on separate sections of colonic tissue. Tissue was washed in bath solution for 5 minutes before and after each 10 minute trial and temperature was maintained at 35°C for the duration of the experiments. Circular muscle contractility was recorded using a Myobath II multi-channel isolated tissue bath system combined with Lab-Trax Data Acquisition System with a 4-channel Transbridge™ amplifier and analyzed using the Data-Trax 2 software (World Precision Instruments, Sarasota, FL). The area under the curve was calculated for all contraction responses. This calculation consisted of subtracting the baseline force (measured in grams) from each data point and then summing the data collected during each 10 minute trial.

Resiniferatoxin (RTX) injections

Prior to injection the animals were anesthetized with isoflurane (1–3% in O_2). Intrathecal injections were made using an 18-gauge needle to make a lumbar puncture between vertebrae L1/L2. A small plastic tube (PE-10; Becton Dickinson, Sparks, MD) was inserted through the needle and the RTX solution (200ng/25 μ l in PBS with 0.25% Tween 80 and 0.05% ascorbic acid) was injected slowly over 2 minutes as previously described (28;30). Following the intrathecal injection animals received an enema of RTX. A small plastic tube (PE-160; Becton Dickinson, Sparks, MD) was inserted 7cm through the anus and the RTX solution (200ng/500 μ l) was infused slowly over 5 minutes. Animals were allowed to recover for two weeks prior to further procedures.

In vivo manometry and colorectal distension

Animals were fasted for 4 hours prior to in vivo recording. While anesthetized with isoflurane (1–3% in O_2) a compliant latex balloon (7cm in length) attached to plastic tubing (PE-160; Becton Dickinson, Sparks, MD) was inserted through the anus and secured in place by taping the tubing to the base of the tail. The distal end of the balloon was at least 1 cm proximal to the external anal sphincter. A second tube for injecting solutions into the lumen of the colon was inserted in parallel to the balloon (PE-160; Becton Dickinson, Sparks, MD). Rats were restrained in a plastic restraining device and the balloon was connected to a pressure transducer (Harvard Apparatus, Holliston, Massachusetts) and Lab-Trax Data Acquisition System with a 4-channel Transbridge™ (World Precision Instruments, Sarasota, FL, USA). Following recovery from anesthesia the animals were allowed 10 minutes to acclimatize before testing began. Colonic contractions (manometry) were recorded at a baseline balloon pressure of 10 mmHg for 10 minutes.

In a separate set of experiments animals were prepared for colorectal distension in a similar manner as for the manometry experiments. The balloon was inflated slowly (0–80 mmHg) to determine the nociceptive threshold. The nociceptive threshold was defined as the balloon pressure that induces a behavioral response such as withdrawal of the testicles, flicking of the tail, vocalization or abdominal contractions (31–33). Colorectal distension was repeated four times with 5 minute intervals and the mean pressures at the nociceptive threshold were recorded for each rat. The PAR-2 agonists SLIGRL (100 μ M; Sigma, St. Louis, MO) or Trypsin (10nM; Invitrogen, Carlsbad, California) (1 ml PBS) were injected into the lumen of the colon over a 2 minute time span using the small plastic injection tube. The investigator

was blinded to whether the rats had been lesioned with resiniferatoxin. Colonic contractions or nociceptive threshold pressures to colorectal distension were recorded before the injection (baseline) and then immediately following the injection of trypsin (0 hour), 3 hours and 6 hours following the injection. With SLIGRL recordings were made prior to injections (baseline) and then immediately following the injection (0 hour) and one hour later. Preliminary experiments demonstrated that the effect of SLIGRL plateaued at 1 hour. This result was consistent with the findings of Kayssi et al. (18). The area under the curve was calculated for all contraction responses. This calculation consisted of subtracting the baseline force (measured in mmHg) from each data point and then summing the data collected during each 10 minute trial.

Immunohistochemistry

Animals were euthanized with pentobarbital (100mg/kg, ip) and perfused through the heart with cold 0.9% saline followed immediately with cold 4% paraformaldehyde in phosphate buffered saline (PBS). DRG (T12-S1) and colon tissue were sectioned at 10µm on a cryostat, placed on a glass slide and air-dried for 1 hour. All preparations were washed 3 times (10 minutes each) in PBS, placed in blocking buffer containing 3% Normal Goat Serum (NGS) with PBS for 1 hour and incubated in primary antibody in 3% NGS/0.3% tween-20/PBS (chicken anti-PAR-2, Aves Labs, Tigard, OR, 1:500; rabbit anti-TRPV1, Affinity Bioreagents, Rockford, IL, 1:500) for 24 hours at 4°C. The sections were then washed 3 times in PBS (10 minutes each) followed by a 1 hour incubation in secondary antibody Alexa Fluor 488 (1:1000; Molecular Probes, Boston, MA) in 3% NGS/0.3% tween-20/PBS. Tissue was then washed 3 times (10 minutes each) and mounted with ProLong® Antifade Kit mounting media (Molecular Probes, Boston, MA) or Vectashield mounting media (Vector Laboratories, Burlingame, CA). The sections were visualized with 485 nm excitation and 530 nm emission filters. Images were photographed on a Leica DM LB2 Fluorescence microscope (Leica, Wetzlar, Germany). Negative controls were prepared by not including the primary antibodies. All images were processed using Adobe Photoshop.

Statistical analysis

All statistics were run using GraphPad Prism version 6. A one-way analysis of variance (ANOVA) with Newman-Keuls Multiple Comparison Test, a two-way ANOVA followed by Bonferroni posttest or a paired t-test was used to analyze the data as appropriate. All values are expressed as means $\pm$ SEM. Data sets were considered significant for $P < 0.05$.

Results

PAR-2 agonists enhance acetylcholine induced colonic contractions in vitro

Acetylcholine (ACh) is known to induce colonic contractions in vitro (34;35), therefore, we used bath applied ACh (200 µM) to stimulate contractions in sections of descending colon. Application of 10 nM trypsin alone to the bath solution did not significantly stimulate contractions as measured by area under the curve (AUC). However, the combination of trypsin and ACh resulted in more sustained and intense contractions than ACh alone. The effects of ACh remained enhanced following the washout of trypsin (One-way ANOVA, $F = 8.148$, $df = 3$, $p = 0.0001$, Newman-Keuls Multiple Comparison Test – ACh vs Trypsin $p < 0.05$; ACh vs ACh + Trypsin $p < 0.05$; ACh vs ACh2 $p < 0.05$) (Figure 1A, B). To verify that the increase in ACh induced contractions were produced by trypsin ACh (200 µM) was applied for 10 minutes, washed out with Tyrodes solution for 20 minutes and then reapplied for 10 minutes. The first application of ACh produced an AUC of 479.3 ± 110.7 and the second application produced an AUC of 350.1 ± 54.8 ($N = 16$ segments of colon, $p = 0.11$ paired t-test). These data indicate that the increased ACh response was due to the trypsin treatment.

Application of the PAR-2 agonist peptide SLIGRL (100 μ M) did not induce colonic contractions when measured by AUC as compared to ACh, which is similar to the effects of trypsin in the assay. When SLIGRL was applied with ACh the contractions were significantly increased as compared to controls. When ACh was re-applied following the washout of SLIGRL the response was significantly larger than the first application of ACh (One-way ANOVA, $F = 9.218$, $df = 3$, $p < 0.0001$, Newman-Keuls Multiple Comparison Test – ACh vs SLIGRL $p < 0.05$; ACh vs ACh + SLIGRL $p < 0.05$; ACh vs ACh2 $p < 0.05$) (Figure 2A, B).

PAR-2 agonists enhance colonic contractions in vivo

To determine the effect of PAR-2 agonists in vivo we used manometry to measure contractions as a function of intraluminal pressure. Luminal pressures from 0–15 mmHg were applied for ten minutes and the corresponding contractile activity of the colon was monitored electronically. Based on these data (not shown) a testing pressure of 10 mmHg was used for subsequent pharmacological studies.

Application of trypsin (10 nM, 1 ml) into the lumen of the colon resulted in an increase in colonic contractions (measured by AUC) at 0, 3 and 6 hours post injection times as compared to the baseline. Animals receiving intraluminal PBS did not demonstrate any change in contractile activity (One-way ANOVA, $F = 19.37$, $df = 3$, $p < 0.0001$, Newman-Keuls Multiple Comparison Test – baseline vs 0 hour $p < 0.01$; baseline vs 1 hour $p < 0.01$; baseline vs 6 hours $p < 0.001$) (Figure 3A,B). Likewise, the administration of SLIGRL (100 μ M, 1 ml) resulted in an increase in colonic contractions at 0 and 1 hour post injection times as compared to the baseline (One-way ANOVA, $F = 50.12$, $df = 2$, $p < 0.0001$, Newman-Keuls Multiple Comparison Test – baseline vs 0 hour $p < 0.001$; baseline vs 1 hour $p < 0.001$) (Figure 4A,B). It was found in preliminary trials that the effects of SLIGRL plateaued within one hour, thus later time points were not collected in these experiments (see Figure 4B).

RTX lesioning suppresses PAR-2 agonist induced colonic contractions, but not PAR-2 agonist induced hypersensitivity to colorectal distension

To eliminate TRPV1 expressing primary afferent neurons rats were injected intrathecally with 200 ng of RTX and another 200 ng of RTX was given as an enema (28). The animals were allowed two weeks to recover following the lesioning procedure. To verify the lesions the DRGs and colons were harvested following the behavioral experiments. Immunohistochemistry was performed on sections of the DRGs and descending colon. Figure 5A demonstrates TRPV1 labeling in the descending colon of a non-lesioned animal. The labeling was similar to that described by Ward et al. (36) and Matsumoto et al. (37;38) and was restricted to neuronal fibers. Enteric neuronal cell bodies were not labeled by the TRPV1 antibody. The tissue nearest the lumen appeared to be labeled most densely, as was previously described (29;38). Lesioning with RTX eliminated the majority of the TRPV1 labeling in the colon (Figure 5B).

PAR-2 labeling in the colon was observed in epithelial cells, enteric neurons and neuronal fibers throughout the colon (Figure 5C)(28;29). In contrast to TRPV1, RTX treatment did not appear to influence PAR-2 labeling of the colon to any significant extent (Figure 5D). This finding is consistent with our previous study (28).

In the thoracic, lumbar and sacral DRGs PAR-2 labeling was eliminated in the lesioned animals (Figure 5E - non-lesioned; 5F - lesioned). These findings are also similar to our previously published results (28).

Administration of trypsin (10 nM, 1 ml) to the lumen of the colon increased colonic contractions (measured by AUC) in non-RTX lesioned animals, but not in lesioned animals (Two-way ANOVA, Interaction $F = 0.9125$, $df = 1$, $p = 0.3556$; RTX Treatment $F = 10.73$, $df = 1$, $p = 0.0055$; Time $F = 0.48$, $df = 1$, $p = 0.4997$; Bonferroni's posttest, Baseline - non-RTX vs RTX $p > 0.05$; 6 hours - non-RTX vs RTX $p < 0.05$) (Figure 6A). The analysis was performed on AUC data, but graphed as percent of control for clarity. However, the RTX lesions had no effect on the trypsin induced hypersensitivity to colorectal distension (One-way ANOVA, $F = 40.98$, $df = 2$, $p < 0.0001$, Newman-Keuls Multiple Comparison Test – Control vs non-RTX + Trypsin $p < 0.001$; Control vs RTX + Trypsin $p < 0.001$; non-RTX + Trypsin vs RTX + Trypsin $p > 0.05$) (Figure 6B). Additionally, the RTX lesion, trypsin treatment or the combination of the two had no effect on compliance in the colon (Two-way ANOVA, Interaction $F = 0.09810$, $df = 3$, $p = 0.9604$; Treatment $F = 1.960$, $df = 1$, $p = 0.1725$) (Figure 6C).

Much like the trypsin injections, non-lesioned animals that received SLIGRL (100 μ M) had an increase in colonic contractions (measured by AUC), but RTX lesioned animals demonstrated a significant decrease in SLIGRL induced motility (Two-way ANOVA, Interaction $F = 6.571$, $df = 1$, $p = 0.0195$; RTX Treatment $F = 6.571$, $df = 1$, $p = 0.0195$; Time $F = 19.05$, $df = 1$, $p = 0.0004$; Bonferroni's posttest – Baseline - non-RTX vs RTX $p > 0.05$; 1 hour - non-RTX vs RTX $p < 0.01$) (Figure 6D). The analysis was performed on AUC data, but graphed as percent of control. Additionally, the RTX lesion did not influence SLIGRL induced hypersensitivity to colorectal distension (One-way ANOVA, $F = 49.13$, $df = 2$, $p < 0.0001$, Newman-Keuls Multiple Comparison Test – Control vs non-RTX + SLIGRL $p < 0.001$; Control vs RTX + SLIGRL $p < 0.001$; non-RTX + SLIGRL vs RTX + SLIGRL $p > 0.05$) (Figure 6E).

Discussion

Function of TRPV1/PAR-2 Colonic Afferent Neurons

In our previous studies we found that there are at least three separate groups of primary afferent neurons that innervate the colon (28;29). One group is derived from the DRGs and express TRPV1 and PAR-2. The second DRG derived group of primary afferent neurons does not express either TRPV1 or PAR-2. The final group is the newly described colospinal afferent neurons. These neurons have their cell bodies in the submucosal and myenteric plexuses and project their axons to the dorsal horn of the lumbar and sacral spinal cord. Colospinal afferent neurons express PAR-2, but not TRPV1. In our earlier studies we found that eliminating the TRPV1 expressing neurons with resiniferatoxin did not alter nociceptive threshold pressures to colorectal distension. We also found that the lesion did not influence the reduction in nociceptive threshold pressures produced by colonic inflammation (28). We verified that there was no TRPV1 present in the colon or in the sacral, lumbar or thoracic DRGs following the lesion. Thus, these data suggested that either the colonic primary afferent neurons that did not express TRPV1 or the colospinal afferent neurons carried mechanical nociceptive information to the central nervous system. In the present study we found that resiniferatoxin lesions blocked PAR-2 agonist induced colonic motility, but did not influence the PAR-2 agonist induced reduction in nociceptive thresholds. Since the lesions eliminated the TRPV1/PAR-2 expressing primary afferent neurons we can conclude that the effects of PAR-2 agonists on colonic motility were mediated by this group of DRG neurons. Our in vitro data supported this finding by demonstrating that PAR-2 agonists alone do not induce contractions in the colon. The agonists required cholinergic input in order to alter motor function. Thus the data indicate that TRPV1/PAR-2 expressing colonic afferent neurons are involved in a motor reflex pathway, but may not be necessary for colonic nociception.

Function of Colospinal Afferent Neurons

The RTX lesioning data indicate that elimination of PAR-2 expressing DRG neurons blocks PAR-2 agonist induced motility in the colon, but does not block PAR-2 agonist induced hypersensitivity to mechanical stimuli. Since the other previously identified group of colonic DRG neurons did not express PAR-2 (28) and intraluminal PAR-2 agonists do not alter colonic compliance (9) (Figure 6C) we can conclude from our data that the novel colospinal afferent neurons are responsible for the PAR-2 agonist effects on mechanical nociception. It is possible that the TRPV1/PAR-2 expressing afferent neurons also transmit nociceptive signals, but their absence does not influence nociceptive thresholds. Thus the data from this study and our previous study indicate that the colospinal afferent neurons conduct mechanical nociceptive information. The data also suggest that inflammation and PAR-2 agonists produce hypersensitivity through these neurons.

Previous Work with TRPV1 in the Colon

Surprisingly, our data conflict with previous reports demonstrating that TRPV1 expressing afferent neurons are critical for mechanical nociception and inflammation induced hypersensitivity in the colon (39–44). In these studies the authors used intraluminal capsaicin to activate TRPV1 fibers in the colon or used capsazepine to block TRPV1 in animals with sensitized colons as a result of inflammation or prior injury. The investigators then used colorectal distension techniques to measure nociceptive threshold pressures. The authors demonstrated that the activation of TRPV1 was associated with decreased nociceptive thresholds. However, Hayashi and colleagues demonstrated that intraluminal administration of capsaicin into the colon of dogs induced giant migrating contractions (45). These contractions were blocked by the muscarinic antagonist atropine and the ganglionic blocker hexamethonium. The giant migrating contractions also required intact innervation of the colon. Hayashi et al.'s work indicates that the TRPV1 expressing primary afferent neurons in the colon are involved in a spinal reflex pathway that probably involves the parasympathetic system. These findings also suggest the possibility that the giant migrating contractions induced by activation of TRPV1 expressing primary afferent neurons may stimulate a separate population of mechano-sensitive afferent neurons. Thus the studies that found that TRPV1 expressing fibers mediated mechanical nociception may have been seeing the indirect nociceptive effects of enhanced colonic motor function rather than a direct effect on mechano-sensitive afferent neurons. Since these authors did not directly test the motor effects of TRPV1 agonists and antagonists in the colon this indirect mechanism would not have been evident (39–44). Alternatively, many of these studies utilized spinal reflex based dependent measures, which we have found to involve TRPV1 expressing primary afferents. These reflexes are likely to be mediated by the same processes as studied by Hayashi and colleagues (45). Thus the investigators may have measured reflex activity rather than colonic nociception in their TRPV1 studies.

Our resiniferatoxin lesioning studies indicate that TRPV1 afferent fibers are not required for detecting mechanical nociception (28). However, the data do not eliminate the possibility that these fibers contribute to nociception either directly or indirectly. Furthermore, our data are consistent with the everyday experience of hundreds of millions of people who consume capsaicin through their intake of chili peppers. In the vast majority of these people there is no gastrointestinal pain associated with capsaicin consumption. Even in naïve individuals, typical capsaicin consumption induces pain only at the oral and anal ends of the gastrointestinal tract suggesting that TRPV1 bearing visceral afferents are not primarily responsible for visceral nociception. It is highly unlikely that people would continue to consume chili peppers if capsaicin induced colonic pain. Interestingly, recent investigations into this issue found that administering capsaicin to the ileum and colon via the stoma in human subjects with ileostomies and colostomies produced pain (46;47). However, the

authors also demonstrated that the capsaicin produced enhanced motor function in the ileum and colon, which may have generated the pain indirectly or translocated the capsaicin back to the stoma to produce the pain via more peripheral receptors. Additionally, the subjects in these studies reported a warming sensation prior to reporting pain suggesting that the relatively high doses of capsaicin may have had some systemic effects. It was previously reported that serosal and mesenteric afferents are particularly responsive to capsaicin and most of these fibers are high-threshold mechano-sensitive afferents that are not responsive to colonic distension (48–50). These fibers are likely nociceptors and could easily be accessed by intraluminally administered small molecules such as capsaicin or its antagonists. Thus the capsaicin in these human subjects may have stimulated the serosal and mesenteric fibers to produce the warming sensation and pain. Intraluminal trypsin or SLIGRL would have difficulty reaching these sites, thus it is not likely that PAR-2 agonists were acting on these neuronal fibers. In contrast to serosal and mesenteric afferents, capsaicin responsive mucosal and submucosal fibers were found to be low-threshold mechano-sensitive and responsive to distension (48–50) and, thus, not likely to be nociceptive fibers. The mucosal environment is readily accessible for both trypsin and SLIGRL, therefore these fibers should be stimulated by the PAR-2 agonists. These mucosal and submucosal fibers likely monitor the chemical environment of the lumen and regulate the movement of the luminal contents. Thus chemical stimuli, such as capsaicin, will evoke a reflex response to hasten elimination of the luminal contents. Our finding that PAR-2 agonists increased colonic motility in vivo and that they enhanced the motor effect of acetylcholine in vitro are consistent with the data on mucosal and submucosal TRPV1 expressing colonic neurons (48–50) and with Hayashi et al's data (45). Combined these data indicate that the TRPV1/PAR-2 expressing colonic afferent neurons are involved in an extrinsic motor reflex pathway. The finding that PAR-2 agonist induced hypersensitivity remains following RTX lesioning suggests a role for colospinal afferent neurons in visceral nociception.

In conclusion, our data indicates that colonic afferent neurons that express TRPV1 and PAR-2 are involved in regulation of motor function and that colospinal afferent neurons transmit nociceptive information in the rat colon. Because colospinal afferent neurons are located in the gut rather than more centrally these neurons are an attractive target for treating visceral pain. It may be possible to suppress their activity with agents that cannot cross the blood brain barrier, thus avoiding many of the side effects of other analgesic agents. Further work is needed to determine if these unique neurons can be selectively targeted for treatment of visceral pain syndromes.

Acknowledgments

This work was supported by the National Institutes of Health NS045614.

SKS – Performed experiments and edited the manuscript.

EMA – Performed experiments and edited the manuscript.

RMC – Wrote the manuscript.

Reference List

1. Dery O, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol*. 1998; 274(6 Pt 1):C1429–C1452. [PubMed: 9696685]
2. Cenac N, Garcia-Villar R, Ferrier L, Larauche M, Vergnolle N, Bunnett NW, et al. Proteinase-activated receptor-2-induced colonic inflammation in mice: Possible involvement of afferent neurons, nitric oxide, and paracellular permeability. *Journal of Immunology*. 2003; 170(8):4296–4300.

3. Cenac N, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, et al. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *American Journal of Pathology*. 2002; 161(5):1903–1915. [PubMed: 12414536]
4. Cenac N, Garcia-Villar R, Mazelin L, Coelho AM, More J, Fioramonti J, et al. Activation of proteinase-activated receptor-2 by the peptide SLIGRL or trypsin induces inflammation of mouse colon. *Gastroenterology*. 2001; 120(5):A725.
5. Coelho AM, Guiard B, Garcia-Villar R, Vergnolle N, Hollenberg MD, Wallace JL, et al. Proteinase-activated receptor-2 (PAR-2) activation produces delayed rectal hyperalgesia in awake rats. *Gastroenterology*. 2001; 120(5):A178.
6. Nguyen C, Coelho AM, Grady E, Compton SJ, Wallace JL, Hollenberg MD, et al. Colitis induced by proteinase-activated receptor-2 agonists is mediated by a neurogenic mechanism. *Canadian Journal of Physiology and Pharmacology*. 2003; 81(9):920–927. [PubMed: 14614531]
7. Reed DE, Barajas-Lopez C, Cottrell G, Velazquez-Rocha S, Dery O, Grady EF, et al. Mast cell tryptase and proteinase-activated receptor 2 induce hyperexcitability of guinea-pig submucosal neurons. *Journal of Physiology-London*. 2003; 547(2):531–542.
8. Kawao N, Ikeda H, Kitano T, Kuroda R, Sekiguchi F, Kataoka K, et al. Modulation of capsaicin-evoked visceral pain and referred hyperalgesia by protease-activated receptors 1 and 2. *Journal of Pharmacological Sciences*. 2004; 94(3):277–285. [PubMed: 15037813]
9. Coelho AM, Vergnolle N, Guiard B, Fioramonti J, Bueno L. Proteinases and proteinase-activated receptor 2: A possible role to promote visceral hyperalgesia in rats. *Gastroenterology*. 2002; 122(4):1035–1047. [PubMed: 11910355]
10. van der Merwe JQ, Ohland CL, Hirota CL, MacNaughton WK. Prostaglandin E-2 Derived from Cyclooxygenases 1 and 2 Mediates Intestinal Epithelial Ion Transport Stimulated by the Activation of Protease-Activated Receptor 2. *Journal of Pharmacology and Experimental Therapeutics*. 2009; 329(2):747–752. [PubMed: 19190238]
11. van der Merwe JQ, Hollenberg MD, MacNaughton WK. EGF receptor transactivation and MAP kinase mediate proteinase-activated receptor-2-induced chloride secretion in intestinal epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2008; 294(2):G441–G451. [PubMed: 18032480]
12. Jarry A, Dorso L, Gratio V, Forgue-Lafitte ME, Laburthe M, Laboisie CL, et al. PAR-2 activation increases human intestinal mucin secretion through EGFR transactivation. *Biochemical and Biophysical Research Communications*. 2007; 364(3):689–694. [PubMed: 18028876]
13. Cuffe JE, Bertog M, Velazquez-Rocha S, Dery O, Bunnett N, Korbmacher C. Basolateral PAR-2 receptors mediate KCl secretion and inhibition of Na⁺ absorption in the mouse distal colon. *Journal of Physiology-London*. 2002; 539(1):209–222.
14. Mall M, Gonska T, Thomas J, Hirtz S, Schreiber R, Kunzelmann K. Activation of ion secretion via proteinase-activated receptor-2 in human colon. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2002; 282(2):G200–G210. [PubMed: 11804840]
15. Mule F, Baffi MC, Falzone M, Cerra MC. Signal transduction pathways involved in the mechanical responses to protease-activated receptors in rat colon. *Journal of Pharmacology and Experimental Therapeutics*. 2002; 303(3):1265–1272. [PubMed: 12438551]
16. Mule F, Baffi MC, Cerra MC. Dual effect mediated by protease-activated receptors on the mechanical activity of rat colon. *British Journal of Pharmacology*. 2002; 136(3):367–374. [PubMed: 12023939]
17. Bian ZX, Li Z, Huang ZX, Zhang M, Chen HL, Xu HX, et al. Unbalanced expression of protease-activated receptors-1 and-2 in the colon of diarrhea-predominant irritable bowel syndrome patients. *Journal of Gastroenterology*. 2009; 44(7):666–674. [PubMed: 19430717]
18. Kayssi A, Amadesi S, Bautista F, Bunnett NW, Vanner S. Mechanisms of protease-activated receptor 2-evoked hyperexcitability of nociceptive neurons innervating the mouse colon. *Journal of Physiology-London*. 2007; 580(3):977–991.
19. Kayssi A, Karanjia R, Bunnett N, Vanner S. Activation of proteinase activated receptor 2 (PAR-2) on nociceptive mouse colonic dorsal root ganglia (DRG) neurons evokes prolonged hyperexcitability and modulates underlying ion channels. *Gastroenterology*. 2005; 128(4):A222.

20. Cenac N, Andrews CN, Holzhausen M, Chapman K, Cottrell G, Andrade-Gordon P, et al. Role for protease activity in visceral pain in irritable bowel syndrome. *J Clin Invest.* 2007; 117(3):636–647. [PubMed: 17304351]
21. Gecse K, Roka R, Ferrier L, Leveque M, Eutamene H, Cartier C, et al. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut.* 2008; 57(5):591–599. [PubMed: 18194983]
22. King CD, Wong F, Currie T, Mauderli AP, Fillingim RB, Riley JL III. Deficiency in endogenous modulation of prolonged heat pain in patients with Irritable Bowel Syndrome and Temporomandibular Disorder. *Pain.* 2009; 143(3):172–178. [PubMed: 19278784]
23. Price DD, Craggs JG, Zhou QQ, Verne GN, Perlstein WM, Robinson ME. Widespread hyperalgesia in irritable bowel syndrome is dynamically maintained by tonic visceral impulse input and placebo/nocebo factors: Evidence from human psychophysics, animal models, and neuroimaging. *Neuroimage.* 2009; 47(3):995–1001. [PubMed: 19375508]
24. Verne GN, Robinson ME, Vase L, Price DD. Reversal of visceral and cutaneous hyperalgesia by local rectal anesthesia in irritable bowel syndrome (IBS) patients. *Pain.* 2003; 105(1–2):223–230. [PubMed: 14499439]
25. Zhou QQ, Fillingim RB, Riley JL, Malarkey WB, Verne GN. Central and peripheral hypersensitivity in the irritable bowel syndrome. *Pain.* 2010; 148(3):454–461. [PubMed: 20074857]
26. Zhou QQ, Fillingim RB, Riley JL, Verne GN. Thermal hypersensitivity in a subset of irritable bowel syndrome patients. *World Journal of Gastroenterology.* 2009; 15(26):3254–3260. [PubMed: 19598301]
27. Dai Y, Moriyama T, Higashi T, Togashi K, Kobayashi K, Yamanaka H, et al. Proteinase-activated receptor 2-mediated potentiation of transient receptor potential vanilloid subfamily 1 activity reveals a mechanism for proteinase-induced inflammatory pain. *J Neurosci.* 2004; 24(18):4293–4299. [PubMed: 15128843]
28. Suckow SK, Caudle RM. NMDA receptor subunit expression and PAR2 receptor activation in colospinal afferent neurons (CANs) during inflammation induced visceral hypersensitivity. *Mol Pain.* 2009; 5:54. [PubMed: 19772634]
29. Suckow SK, Caudle RM. Identification and immunohistochemical characterization of colospinal afferent neurons in the rat. *Neuroscience.* 2008; 153(3):803–813. [PubMed: 18424003]
30. Karai L, Brown DC, Mannes AJ, Connelly ST, Brown J, Gandal M, et al. Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. *J Clin Invest.* 2004; 113(9):1344–1352. [PubMed: 15124026]
31. Ness TJ, Gebhart GF. Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudoreflexes in the rat. *Brain Res.* 1988; 450(1–2):153–169. [PubMed: 3401708]
32. Wesselmann U, Czakanski PP, Affaitati G, Giamberardino MA. Uterine inflammation as a noxious visceral stimulus: behavioral characterization in the rat. *Neurosci Lett.* 1998; 246(2):73–76. [PubMed: 9627183]
33. Zhou Q, Price DD, Caudle RM, Verne GN. Visceral and somatic hypersensitivity in a subset of rats following TNBS-induced colitis. *Pain.* 2008; 134(1–2):9–15. [PubMed: 17481818]
34. Harrison JS, McSwiney BA. The chemical transmitter of motor impulses to the stomach. *J Physiol.* 1936; 87(1):79–86. [PubMed: 16994779]
35. Paton WD, Zar MA. The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. *J Physiol.* 1968; 194(1):13–33. [PubMed: 4295753]
36. Ward SM, Bayguinov J, Won KJ, Grundy D, Berthoud HR. Distribution of the vanilloid receptor (VR1) in the gastrointestinal tract. *Journal of Comparative Neurology.* 2003; 465(1):121–135. [PubMed: 12926020]
37. Matsumoto K, Kurosawa E, Terui H, Hosoya T, Tashima K, Murayama T, et al. Localization of TRPV1 and contractile effect of capsaicin in mouse large intestine: high abundance and sensitivity in rectum and distal colon. *Am J Physiol Gastrointest Liver Physiol.* 2009
38. Matsumoto K, Hosoya T, Tashima K, Namiki T, Murayama T, Horie S. Distribution of Transient Receptor Potential Vanilloid 1 Channel-Expressing Nerve Fibers in Mouse Rectal and Colonic

- Enteric Nervous System: Relationship to Peptidergic and Nitrergic Neurons. *Neuroscience*. 2011; 172:518–534. [PubMed: 20951772]
39. van den Wijngaard RM, Klooker TK, Welting O, Stanisor OI, Wouters MM, van der CD, et al. Essential role for TRPV1 in stress-induced (mast cell-dependent) colonic hypersensitivity in maternally separated rats. *Neurogastroenterol Motil*. 2009; 21(10):1107–1e94. [PubMed: 19523146]
 40. Christianson JA, McIlwrath SL, Koerber HR, Davis BM. Transient receptor potential vanilloid 1-immunopositive neurons in the mouse are more prevalent within colon afferents compared to skin and muscle afferents. *Neuroscience*. 2006; 140(1):247–257. [PubMed: 16564640]
 41. Jones RC III, Xu L, Gebhart GF. The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J Neurosci*. 2005; 25(47):10981–10989. [PubMed: 16306411]
 42. Miranda A, Nordstrom E, Mannem A, Smith C, Banerjee B, Sengupta JN. The role of transient receptor potential vanilloid 1 in mechanical and chemical visceral hyperalgesia following experimental colitis. *Neuroscience*. 2007; 148(4):1021–1032. [PubMed: 17719181]
 43. Qin HY, Luo JL, Qi SD, Xu HX, Sung JJ, Bian ZX. Visceral hypersensitivity induced by activation of transient receptor potential vanilloid type 1 is mediated through the serotonin pathway in rat colon. *Eur J Pharmacol*. 2010; 147(6):789–797. [PubMed: 20079916]
 44. Winston J, Shenoy M, Medley D, Naniwadekar A, Pasricha PJ. The vanilloid receptor initiates and maintains colonic hypersensitivity induced by neonatal colon irritation in rats. *Gastroenterology*. 2007; 132(2):615–627. [PubMed: 17258716]
 45. Hayashi K, Shibata C, Nagao M, Sato M, Kakyo M, Kinouchi M, et al. Intracolonic capsaicin stimulates colonic motility and defecation in conscious dogs. *Surgery*. 2010; 147(6):789–797. [PubMed: 20079916]
 46. Arendt-Nielsen L, Schipper KP, Dimcevski G, Sumikura H, Krarup AL, Giamberardino MA, et al. Viscero-somatic reflexes in referred pain areas evoked by capsaicin stimulation of the human gut. *European Journal of Pain*. 2008; 12(5):544–551. [PubMed: 17977761]
 47. Drewes AM, Schipper KP, Dimcevski G, Petersen P, Gregersen H, Funch-Jensen P, et al. Gut pain and hyperalgesia induced by capsaicin: a human experimental model. *Pain*. 2003; 104(1–2):333–341. [PubMed: 12855343]
 48. Lynn PA, Blackshaw LA. In vitro recordings of afferent fibres with receptive fields in the serosa, muscle and mucosa of rat colon. *Journal of Physiology-London*. 1999; 518(1):271–282.
 49. Song XY, Chen BN, Zagorodnyuk VP, Lynn PA, Blackshaw LA, Grundy D, et al. Identification of Medium/High-Threshold Extrinsic Mechanosensitive Afferent Nerves to the Gastrointestinal Tract. *Gastroenterology*. 2009; 137(1):274–284. [PubMed: 19268671]
 50. Brierley SM, Carter R, Jones W, Xu LJ, Robinson DR, Hicks GA, et al. Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice. *Journal of Physiology-London*. 2005; 567(1):267–281.

Figure 1.

Trypsin enhances the effects of acetylcholine on contraction in segments of colon in vitro. Sections of descending rat colon (2cm) were placed into a warmed bath as described in the methods. Trypsin (10nM) and acetylcholine (ACh) (200 μ M) were applied via the bath solution as indicated for 10 minutes. The tissue was washed with fresh solution between each agent and recordings were made during this time to ensure that the tissue recovered back to baseline levels. **A.** A representative experiment demonstrating the effects of trypsin and ACh on contraction in a segment of colon. **B.** Summary of area under the curve (AUC) data for each treatment. Asterisks indicate $p < 0.05$, one-way ANOVA followed by Newman-Keuls Multiple Comparison Test when compared to the first ACh treatment. (N = 16 colon segments from eight rats)

Figure 2. SLIGRL enhances the effects of acetylcholine on contraction in segments of colon in vitro. Sections of descending rat colon (2cm) were placed into a warmed bath as described in the methods. SLIGRL (100 μ M) and acetylcholine (ACh) (200 μ M) were applied via the bath solution as indicated for 10 minutes. The tissue was washed with fresh solution between each agent and recordings were made during this time to ensure that the tissue recovered back to baseline levels. **A.** A representative experiment demonstrating the effects of SLIGRL and ACh on contraction in a segment of colon. **B.** Summary of area under the curve (AUC) data for each treatment. Asterisks indicate $p < 0.05$, one-way ANOVA followed by Newman-Keuls Multiple Comparison Test when compared to the first ACh treatment. (N = 16 colon segments from 8 rats)

Figure 3.

Trypsin administered intraluminally enhances contractions in the colon of rats. Manometry was performed on rats as described in the methods section. Briefly, a balloon was placed into the colon and inflated to 10 mmHg. Fluctuations in pressure were then monitored prior to the administration of 1 ml of 10nM trypsin, immediately after the trypsin (0 hour), and 1 hour and 6 hours after the trypsin. **A.** Representative recordings from one rat at 0 and 6 hours after trypsin administration. **B.** Summary of the area under the curve (AUC) data for trypsin on colonic contractility. Single asterisk indicates $p < 0.01$ and two asterisks indicate $p < 0.001$, one-way ANOVA followed by Newman-Keuls Multiple Comparison. (N = 5 rats)

Figure 4. SLIGRL administered intraluminally enhances contractions in the colon of rats. Manometry was performed on rats as described in the methods section. Briefly, a balloon was placed into the colon and inflated to 10 mmHg. Fluctuations in pressure were then monitored prior to the administration of 1 ml of 100 μ M SLIGRL, immediately after the SLIGRL (0 hour) and 1 hour after the SLIGRL. **A.** Representative recordings from one rat at 0 and 1 hour after SLIGRL administration. **B.** Summary of the area under the curve data for SLIGRL on colonic contractility. Asterisk indicates $p < 0.01$, one-way ANOVA followed by Newman-Keuls Multiple Comparison. (N = 5 rats)

Figure 5.

Effect of resiniferatoxin lesioning on TRPV1 labeling in the colon and PAR-2 labeling in the colon and DRG. Rats received intrathecal injections of resiniferatoxin (200ng) and enemas of resiniferatoxin (200ng), and the tissue was prepared for immunohistochemistry as described in the methods. Control animals did not receive any treatment. **A.** Representative section of colon demonstrating TRPV1 labeling in a non-lesioned animal. Arrows indicate examples of TRPV1 labeled neuronal fibers. **B.** TRPV1 labeling in the colon of a lesioned animal. **C.** PAR-2 labeling in the colon of a non-lesioned animal. Arrows indicate examples of PAR-2 labeled neuronal cell bodies. **D.** PAR-2 labeling in the colon of a lesioned animal. Arrows indicate examples of PAR-2 labeled neuronal cell bodies. **E.** PAR-2 labeling in a lumbar DRG of a non-lesioned rat. **F.** PAR-2 labeling in a lumbar DRG of a lesioned rat. Scale bars = **A.** 50 μ m, **E.** 20 μ m.

Figure 6.

Resiniferatoxin lesions suppress PAR-2 agonist effects on contraction, but not on nociceptive thresholds. Resiniferatoxin lesioned animals were prepared as described in the methods. Control animals received no treatment. **A.** Effect of intraluminal trypsin (10nM, 1ml) on colonic contractions, as measured by manometry in vivo, for resiniferatoxin (RTX) lesioned ($N = 8$) and non-lesioned animals ($N = 7$). Asterisk indicate $p < 0.05$, Two-way ANOVA followed by Bonferroni's posttest when comparing lesioned and non-lesioned animals at 6 hours. The analysis was performed on AUC data, but graphed as percent of control. **B.** Effect of intraluminal trypsin (10nM, 1ml) on nociceptive thresholds in RTX lesioned ($N = 8$) and non-lesioned animals ($N = 7$). Asterisks indicate $p < 0.05$, one-way ANOVA followed by Newman-Keuls Multiple Comparison Test when compared to control thresholds obtained in naïve rats ($N = 10$). The analysis was performed on AUC data, but graphed as percent of control. **C.** Effect of intraluminal trypsin (10nM, 1 ml) on colonic compliance. Pressure versus volume relationships were generated via a balloon inserted into the rectum/colon in RTX lesioned ($N = 5$) and non-lesioned ($N = 6$) rats before and 6 hours following intraluminal trypsin. RTX lesioning, trypsin treatment or a combination of both treatments had no effects on compliance (Two-way ANOVA, $p > 0.05$). **D.** Effect of intraluminal SLIGRL (100μM, 1ml) on colonic contractions, as measured by manometry in vivo, for RTX lesioned ($N = 5$) and non-lesioned animals ($N = 6$). Asterisk indicate $p < 0.05$, Two-way ANOVA followed by Bonferroni's posttest when comparing lesioned and non-lesioned animals at 1 hour. **E.** Effect of intraluminal SLIGRL (100μM, 1ml) on nociceptive thresholds in resiniferatoxin lesioned ($N = 4$) and non-lesioned animals ($N = 4$). Asterisks indicate $p < 0.05$, one-way ANOVA followed by Newman-Keuls Multiple Comparison Test when compared to control thresholds obtained in naïve rats ($N = 10$).