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CaM kinase II in colonic smooth muscle contributes to dysmotility in murine DSS-colitis

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Abstract

Altered calcium mobilization has been implicated in the development of colonic dysmotility in inflammatory bowel disease. The aim of this study was to investigate the mechanisms by which disrupted intracellular Ca^{2+} signaling contributes to the impaired contractility of colon circular smooth muscles. Acute colitis was induced in C57Bl/6 mice with dextran sulfate sodium (DSS) in the drinking water for five days. Spontaneous and acetylcholine-evoked contractions, caffeine-evoked hyperpolarization, and SERCA2 and phospholamban expression were reduced compared to controls. Tetrodotoxin did not restore control levels of contractile activity. The amplitudes, but not the frequency, of intracellular Ca^{2+} waves were increased compared to controls. Caffeine abolished intracellular Ca^{2+} waves in control smooth muscle cells, but not in smooth muscle cells from DSS-treated mice. CaM kinase II activity and cytosolic levels of HDAC4 were increased, and $\text{I}\kappa\text{B}\alpha$ levels were decreased in distal colon smooth muscles from DSS-treated mice. These results suggest that disruptions in intracellular Ca^{2+} mobilization due to down-regulation of SERCA2 and phospholamban expression lead to increased CaM kinase II activity and cytosolic HDAC4 that may contribute to the dysmotility of colonic smooth muscles in colitis by enhancing NF- κB activity.

Keywords

Ca^{2+} waves; CaM kinase II; colonic smooth muscle; DSS colitis; phospholamban; SERCA

Ulcerative colitis and Crohn's disease are inflammatory bowel diseases that involve chronic intestinal inflammation associated with dysmotility resulting in symptoms that include abdominal pain, weight loss, and bloody diarrhea [1-3]. Inflammation is associated with reduced colonic mixing patterns and impaired haustra formation, but increased propulsive motility from propagating contractions, causing rapid movement of the colonic contents to the rectum, accentuating the diarrhea [4-7]. Multiple mechanisms involving changes in enteric neurotransmission, afferent sensory input, and intrinsic smooth muscle contractility underlie the dysmotility [5].

Improper Ca^{2+} mobilization is implicated in the dysmotility of colon circular smooth muscle cells from ulcerative colitis patients and in animal models of colitis [8;9]. Impaired Ca^{2+} influx in smooth muscles from the inflamed colon has been reported to occur by reduced L-type Ca^{2+} channel expression or by decreased L-type Ca^{2+} channel activity with no change

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in expression [10;11]. In addition, SERCA2 expression in rat colon smooth muscle is decreased in colitis, suggesting that reduced SR Ca^{2+} uptake can also disrupt intracellular Ca^{2+} mobilization in smooth muscles from inflamed colon [12]. A variety of effectors, including Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and Ca^{2+} -sensitive ion channels are regulated by SR Ca^{2+} dynamics [13-15]. HDAC4 is sequestered in the cytoplasm following CaMKII phosphorylation, allowing HDAC4 target genes to be de-repressed [16]. NF- κ B-dependent gene expression requires transcriptional co-activators, including histone acetyltransferases (HATs) and histone deacetylases (HDACs) [17]. These findings suggest that CaMKII could play a role in colonic dysmotility by enhancing NF- κ B-dependent gene transcription.

Understanding the molecular mechanisms underlying the disruptions in Ca^{2+} mobilization and the impaired contractile responses of colon smooth muscles to intestinal inflammation is important for understanding the myogenic basis of the dysmotility that is associated with colitis. In the present study, we assessed SR Ca^{2+} dynamics by evaluating intracellular Ca^{2+} wave properties and CaMKII activity following DSS-induced colitis. SERCA2, PLB, and γ CaMKII protein expression, and cytosolic levels of HDAC4 and I κ B α were also examined.

Methods

Inducing DSS-colitis

Male C57Bl/6 mice (6-8 week old, Charles River Laboratories, Wilmington, MA) were administered 3.5% w/v DSS (MW = 36,000-50,000 Da, MP Biomedicals LLC, Solon, OH) *ad libitum* in the drinking water for five days. Distal colons were utilized up to five days post-DSS treatment [18]. Mice were maintained and experiments carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All protocols were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

Preparation of distal colon smooth muscles

Mice were anesthetized with isofluorane and euthanized by decapitation. The colons were removed, pinned to a Sylgard-lined dish containing oxygenated Krebs solution, and the mucosa and submucosa were removed by sharp dissection. The distal colon was identified as a 2 cm region beginning 0.5 cm aboral from the proximal colon mucosal striations.

Histological staining

Distal colon cross sections (5 μ m) were fixed in 10% v/v neutral buffered formalin solution, embedded in paraffin, and H&E stained for light microscopy.

CaMKII assays

Total (Ca^{2+} /CaM stimulated) and autonomous (Ca^{2+} /CaM independent) CaMKII activity in distal colon smooth muscles were assayed using the specific CaMKII peptide substrate autocalyxin-2 (KKALRRQETVDAL, 20 μ M, BioMol, Plymouth Meeting, PA) [19].

SDS-PAGE and western blotting

The cytosolic and SR fractions were obtained from distal colon smooth muscles frozen in liquid nitrogen, and stored at -80°C [20]. Protein concentrations were determined by Bradford assay using bovine γ -globulin as standard. Cytosolic fractions were analyzed with anti- γ CaMKII, HDAC4, and I κ B α antibodies, and SR fractions were analyzed with anti-SERCA2, and PLB antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunostained protein bands were detected using ECL Advantage (GE HealthCare Biosciences,

Piscataway, NJ), and visualized with a CCD camera-based detection system (Epi Chem II, UVP Laboratory Products, Upland, CA). The .tiff images were analyzed with Adobe Photoshop (CS2, V9.0.2, Adobe Systems, San Jose, CA) and densitometry was carried out using Un-Scan-It software (Silk Scientific, Orem, UT).

Co-immunoprecipitation

γ CaMKII was immunoprecipitated from cytosolic fractions with anti- γ CaMKII antibodies and Protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The beads were boiled in SDS-sample buffer, and the bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with anti-HDAC4 antibodies, and visualized by enhanced chemiluminescence as described above.

Mechanical responses

Contractile activity from control and DSS-colitis smooth muscle strips was measured using standard myobath techniques, with each strip attached to a Fort 10 isometric strain gauge (WPI, Sarasota, FL) in parallel with the circular muscles [20]. A resting force of 6 mN was applied and tissues were equilibrated for ~ 1 h. Responses were recorded using Acqknowledge 3.2.7 software (BIOPAC Systems).

Intracellular microelectrode recordings

Distal colon smooth muscles from control and DSS-colitis mice were perfused with Krebs ($37.5 \pm 0.5^\circ\text{C}$, pH 7.3-7.4) bubbled with 97% O_2 -3% CO_2 and 1 μM nicardipine (to control tissue movement) [20]. Smooth muscle cells were impaled with glass microelectrodes filled with 3M KCl having resistances between 50-80 M Ω . Transmembrane potential was measured with a high input impedance amplifier (model S-7071, WPI). Electrical signals were recorded with Axoscope 9.2 data acquisition software (Axon Instruments, Union City, CA), and printed using Clampfit analysis software (Axon Instruments).

Visualization and image analysis of intracellular Ca^{2+} waves

Distal colon smooth muscles were pinned down with longitudinal muscle facing up in a bath containing oxygenated Krebs at $36.5 \pm 0.5^\circ\text{C}$. The tissue was equilibrated for 1 h, incubated with 10 μM fluo-4 AM (Molecular Probes, Eugene, OR) for 20 min at room temperature, and then perfused with oxygenated Krebs for 15-20 min at $36.5 \pm 0.5^\circ\text{C}$ to allow for de-esterification [15;21;22]. Intracellular Ca^{2+} waves were recorded from $150 \mu\text{m} \times 150 \mu\text{m}$ regions of interest (ROIs) of intact smooth muscles from day 4 post-DSS treatment and from age-matched control mice [22]. Exposure matching was used to compare control and drug effects because subsequent exposure of fluo-4AM to the excitation light will decrease the amplitude and frequency of the intracellular waves [15]. Image sequences were analyzed with custom software (Volumetry G6a, by G.W. Hennig) to generate spatiotemporal (ST) maps of intracellular Ca^{2+} waves [21]. Frequency (Hz), amplitude (intensity units, *iu*), and area under the curve (AUC) of waves from individual cells were calculated [21]. The amplitudes of intracellular Ca^{2+} waves were measured using the F/F_{avg} ratio as intensities of Ca^{2+} -induced fluorescence was constantly oscillating, preventing F_0 from being determined [15;21].

Materials

Tetrodotoxin (TTX), 2-aminoethoxydiphenylborate (2-APB), and acetylcholine (ACh) were obtained from Sigma (St. Louis, MO), caffeine from Calbiochem (La Jolla, CA), and western blot materials, including nitrocellulose paper, gel components, and Bradford assay materials from BioRad (Hercules, CA). Paraffin, alcohols, dyes, and xylene for histological staining were purchased from Richard Allan Scientific (Kalamazoo, MI).

Statistical Analysis

Averages from experiments were analyzed for statistical significance using Sigma Stat software (Access Softek Inc.). Mouse weights, contractile data, and CaMKII activity before and after DSS treatment were analyzed by One-Way ANOVA and data are expressed as means $\pm$ SD. Western blot data was normalized to controls or to the average of controls and tested for significance with *t*-test. Intracellular wave data were analyzed with One-Way ANOVA, data are expressed as means $\pm$ SEM, and $P < 0.05$ was considered significant.

Results

Mechanical responses

The frequencies of spontaneous phasic contractions of muscle strips from control and DSS-colitis mice were not significantly different (0.5 ± 0.1 cycles per minute (cpm) vs. 0.4 ± 0.07 cpm, respectively, $n=7$, $P > 0.05$). The amplitudes of spontaneous phasic contractions of muscle strips from mice with DSS-colitis were significantly decreased (2.7 ± 0.8 mN vs. 1.6 ± 0.4 mN, for control and DSS-colitis mice, respectively, $n=7$, $P < 0.05$) (Figure 1A). ACh ($1\mu\text{M}$) increased the amplitudes of phasic contractions of control smooth muscle strips from 2.7 ± 0.8 mN to 6.9 ± 0.4 mN ($n=5$, $P < 0.001$), and also increased the frequency of phasic contractions from 0.5 ± 0.1 cpm to 0.8 ± 0.1 cpm ($n=6$, $P < 0.001$) (Figure 1B). ACh increased the amplitudes of phasic contractions of smooth muscle strips from DSS-colitis mice from 1.6 ± 0.4 mN to 3.6 ± 1.3 mN ($n=7$, $P < 0.001$), but did not significantly increase the frequency of spontaneous contractions (0.4 ± 0.07 cpm vs 0.5 ± 0.1 cpm $n=7$, $P > 0.05$) (Figure 1B). The amplitude of 3.6 ± 1.3 mN from DSS-affected colons is significantly lower than the amplitude of 6.9 ± 0.4 mN from control smooth muscle strips in the presence of $1\mu\text{M}$ ACh ($P < 0.001$).

TTX increased the amplitudes of phasic contractions of control smooth muscles from 2.7 ± 0.8 mN to 3.6 ± 1.9 mN ($n=8$, $P < 0.05$) and increased the frequency of phasic contractions from 0.5 ± 0.1 cpm to 0.8 ± 0.1 cpm ($n=5$, $P < 0.001$) (Figure 1C). The amplitudes of the phasic contractions of smooth muscle strips from DSS-colitis mice were not significantly increased by TTX (1.6 ± 0.4 mN vs. 1.5 ± 0.4 mN in the absence and presence of TTX, respectively, $n=8$) (Figure 1C). The amplitude of 1.5 ± 0.4 mN from DSS-affected colons is significantly lower than the amplitude of 3.6 ± 1.9 mN from control smooth muscle strips ($P < 0.05$). TTX increased the frequency of phasic contractions in DSS-colitis mice from 0.4 ± 0.07 cpm to 0.6 ± 0.04 cpm ($n=7$, $P < 0.05$). The frequency of 0.6 ± 0.04 cpm from DSS-affected colons is significantly lower than the frequency of 0.8 ± 0.1 cpm from control smooth muscle strips in the presence of TTX ($P < 0.05$).

Effect of caffeine on intracellular Ca^{2+} waves

The frequency, amplitude, and AUC of Ca^{2+} waves were measured from 3-4 individual myocytes from 5 separate preparations (Figure 2A, B). The frequencies of Ca^{2+} waves in myocytes from control or DSS-colitis mice were not significantly different (1.1 ± 0.06 Hz and 1.1 ± 0.06 Hz, respectively, $n=15$, $P > 0.05$) (Figure 2C, D, and G). The amplitudes and AUC of Ca^{2+} waves in myocytes from mice with DSS-colitis were increased by 33% (32 ± 5 *iu* vs. 24 ± 3 *iu*, $n=15$, $P > 0.05$), and 50% (12 ± 2 *iu* vs. 8 ± 1 *iu*, $n=15$, $P > 0.05$), respectively compared to control mice (Figure 2H, I). Additional differences in Ca^{2+} waves became apparent in the presence of 1mM caffeine. The Ca^{2+} wave frequencies were decreased by 71% (1.1 ± 0.06 Hz to 0.3 ± 0.01 Hz, $n=15$, $P < 0.001$), and 39% (1.1 ± 0.06 Hz to 0.7 ± 0.04 Hz, $n=15$, $P < 0.001$), in control myocytes, and myocytes from mice with DSS-colitis, respectively (Figure 2E, F, and G). The frequency of 0.7 ± 0.04 Hz is significantly greater than the frequency of 0.3 ± 0.01 Hz ($P < 0.001$) from controls. Caffeine reduced the amplitudes of Ca^{2+} waves by 79% in control myocytes, (24 ± 3 *iu* to 5 ± 1 *iu*,

$n=15$, $P < 0.001$), and by 50% in myocytes from mice with DSS-colitis (32 ± 5 *iu* to 16 ± 3 *iu*, $n=15$, $P < 0.05$). The amplitude of 16 ± 3 *iu* is significantly greater than the amplitude of 5 ± 1 *iu* from controls ($P < 0.001$) (Figure 2H). Caffeine reduced the AUC of Ca^{2+} waves by 62% in control myocytes (8 ± 1 *iu* to 3 ± 1 *iu*, $n=15$, $P < 0.05$), and by 42% in myocytes from mice with DSS-colitis (12 ± 2 *iu* to 7 ± 1 *iu*, $n=15$, $P < 0.05$). The AUC of 12 ± 2 *iu* is significantly greater than the AUC of 7 ± 1 *iu* from controls ($P < 0.05$) (Figure 2I). Ca^{2+} waves were abolished by 2-APB (30 μM) but were unaffected by ryanodine (10 μM) (*data not shown*), indicating that intracellular Ca^{2+} waves are mediated by inositol 1,4,5-trisphosphate receptors (IP_3R) [23].

Effect of caffeine on RMP

Intracellular Ca^{2+} waves regulate membrane potential and contribute to smooth muscle excitability [23;24]. Therefore, we examined the RMP in the presence of 1mM caffeine in control and DSS-colitis smooth muscles. The effects of caffeine were fully reversible upon washout and were insensitive to 0.3 μM TTX (*data not shown*). The RMPs of smooth muscle cells from control (-46.6 ± 3 mV, $n=10$) and DSS-colitis mice (-47.3 ± 2 mV, $n=14$) were not significantly different ($P > 0.05$). Caffeine induced a larger hyperpolarization (ΔRMP) in smooth muscles from DSS-colitis mice compared to controls (16.0 ± 2 mV vs. 12 ± 1 mV, $n=6$, $*P < 0.05$) (Figure 3A, B).

DSS-colitis decreases SERCA2 and PLB expression

The results shown in Figures 2 and 3 suggest that DSS-colitis alters SR Ca^{2+} mobilization in distal colon smooth muscles. SERCA2 and PLB expression progressively decreased in smooth muscles from DSS-colitis mice (Figure 4A, C). SERCA2 expression became significantly lower than control at day 2, and reached plateau values 30%-35% of control at days 4 and 5 post-DSS treatment (Figure 4B) ($n=5$, $*P < 0.05$). PLB expression became significantly lower than control at day 3, and reached plateau values 50%-60% of control at days 4 and 5 post-DSS treatment (Figure 4D) ($n=5$, $*P < 0.05$). Smooth muscle myosin heavy chain expression was unaffected by DSS-colitis (lower panels, Figure 4A, C; 5 μg protein per lane).

DSS-colitis increases CaMKII activity and expression

Increased intracellular Ca^{2+} wave frequencies in antrum smooth muscles activates CaMKII [15]. Since DSS-colitis increased the amplitudes and AUC of Ca^{2+} waves (Figure 2), CaMKII activity in distal colon smooth muscles from control, and DSS-colitis mice was measured. CaMKII is activated in smooth muscles from DSS-colitis mice, as indicated by the increased autonomous activity on days 1-5 post-DSS treatment compared to controls (Figure 5A) ($n=7$, $*P < 0.05$). Unexpectedly, total CaMKII activity was also increased on days 1-5 post-DSS treatment (Figure 5B), suggesting that CaMKII expression is increased ($n=7$, $*P < 0.05$) [25]. Western blot and densitometry analysis shows that γCaMKII expression was significantly increased in distal colon smooth muscles from mice with DSS-colitis on days 1-5 post-DSS treatment compared to controls (Figure 5C, D).

DSS-colitis decreases cytosolic I $\kappa\text{B}\alpha$ levels

Pro-inflammatory cytokines activate NF- κB by proteolysis of the inhibitory I $\kappa\text{B}\alpha$ subunit [26]. Thus, decreased levels of I $\kappa\text{B}\alpha$ are an indicator of NF- κB activation [27]. Western blot and densitometry analysis shows that cytosolic I $\kappa\text{B}\alpha$ levels were significantly decreased compared to control levels (Figure 6A, B; $n=4$, $*P < 0.05$) on days 3-5 post-DSS treatment.

CaMKII increases cytosolic HDAC4 levels in DSS-colitis

The findings that DSS-colitis elevated CaMKII activity and expression in distal colon smooth muscles prompted us to examine a CaMKII substrate that is a key component of inflammatory signaling pathways. HDAC4 translocates to the cytoplasm following phosphorylation by CaMKII, neutralizing the suppression of NF- κ B-dependent gene transcription by HDAC4 [16]. Western blot and densitometry analysis shows that cytosolic HDAC4 levels were significantly increased compared to controls (Figure 7A, B; $n=4$, $*P < 0.05$) on days 1-5 post-DSS treatment, when CaMKII activity and expression was also increased (Figure 5). HDAC4 phosphorylation by CaMKII depends on the binding of activated CaMKII to HDAC4 [27]. Co-immunoprecipitation experiments show that γ CaMKII binding to HDAC4 was increased on day 3 post-DSS treatment (Figure 7C), when HDAC4 cytosolic levels and CaMKII autonomous activity were also increased.

Discussion

We used the murine DSS-colitis model to investigate the mechanisms by which disrupted intracellular Ca^{2+} signaling contributes to the impaired contractility of colon circular smooth muscles. Although the DSS-colitis model is generally considered an acute injury model, the colitis shares clinical and histopathologic characteristics with ulcerative colitis [28;29]. The responsiveness of the DSS-colitis model to sulphasalazine, olsalazine, and recently infliximab, further supports its utility as a model of ulcerative colitis [29]. The reduced contractility of colon smooth muscles in animal models of colitis is well documented [3;5;7;8;10;30;31]. Similarly, we found that the spontaneous phasic contractions and ACh-evoked contractions of distal colon circular smooth muscles were diminished by DSS-colitis. The neuronal blocker TTX increased the frequency of spontaneous phasic contractions of smooth muscle strips from DSS-colitis mice, suggesting the presence of ongoing inhibitory neural activity. However, TTX did not increase the amplitudes of spontaneous or ACh-evoked contractions, indicating the presence of a myogenic defect independent of any neurogenic defect. Recent findings showing increased carbachol-evoked contractions of distal colon circular smooth muscles from DSS-treated female Balb/c mice are in contrast to these findings, and may reflect differences due to the time and dose of DSS administered, and strain and gender differences in the manifestation of DSS-colitis [32].

The mechanisms by which impaired calcium mobilization contributes to the reduced contractility of smooth muscles from ulcerative colitis patients, and animal models of colitis are not fully understood [8;9;12;33]. Reduced activity and expression of L-type Ca^{2+} channels, or interference with Ca^{2+} release from intracellular stores has been suggested [9;11;12]. In this study we measured intracellular Ca^{2+} waves to investigate SR Ca^{2+} mobilization. We found that the Ca^{2+} wave frequencies in control strips and strips from mice with DSS-colitis were essentially identical. In contrast, the amplitudes and the AUCs were increased (33% and 50% respectively) in distal colon smooth muscles from DSS-treated mice, although statistical analyses indicated these increases were not significant. The lack of statistical significance may be due to variability in wave amplitudes and AUCs observed between individual mice, as well as differences in basal Ca^{2+} levels which could affect the dynamic range of the Ca^{2+} dye. Since caffeine diminishes intracellular Ca^{2+} wave activity by SR Ca^{2+} store depletion, we examined the effect of caffeine on Ca^{2+} waves [21]. Caffeine decreased Ca^{2+} wave activity in smooth muscles from untreated mice, as indicated by reductions of 71%, 79%, and 62%, respectively, in the frequency, amplitude, and AUC of intracellular Ca^{2+} waves. In contrast, the frequency, amplitude, and AUC of Ca^{2+} waves in distal colon smooth muscles from DSS-colitis mice were decreased by 39%, 50%, and 42%, respectively, indicating that intracellular Ca^{2+} mobilization is altered by DSS-colitis. Intracellular Ca^{2+} waves result from the spatio-temporal recruitment of Ca^{2+} puffs from IP_3Rs [21;23]. Our findings (data not shown) that 2-APB, but not ryanodine, abolished

intracellular Ca^{2+} waves support the conclusion that the waves originate from IP_3R -mediated Ca^{2+} release. IP_3Rs are stimulated at low Ca^{2+} concentrations ($< 300\text{nM}$) but inhibited by higher Ca^{2+} concentrations ($> 300\text{nM}$) [34]. The increased amplitude and AUC of Ca^{2+} waves, and persistence of waves in the presence of caffeine suggest that the altered Ca^{2+} mobilization in smooth muscles from mice with DSS colitis may elevate basal cytosolic Ca^{2+} concentration to within the range that activates IP_3Rs . We measured the effects of caffeine on membrane potential and contraction to examine this possibility. Caffeine hyperpolarized smooth muscles from control, and DSS-colitis mice by 16mV and 12mV, respectively, supporting the idea that the persistence of Ca^{2+} waves in the presence of caffeine blunted the hyperpolarization, in contrast to control smooth muscles in which Ca^{2+} wave activity was markedly reduced. It is unclear how a reduction in Ca^{2+} waves could result in hyperpolarization, since IP_3 -mediated SR Ca^{2+} release events (Ca^{2+} puffs) increase spontaneous transient outward currents (STOCs) and hyperpolarize colon smooth muscle cells [23;35;36]. Decreased intracellular Ca^{2+} wave activity may also result in a reduction in the activities of Ca^{2+} -sensitive non-selective cation channels, facilitating hyperpolarization, although additional studies are necessary to test this possibility.

TNBS-colitis reduces SERCA2 expression in rat colon smooth muscle, suggesting that an altered balance between cytoplasmic Ca^{2+} and SR Ca^{2+} contributes to the defects in intracellular Ca^{2+} mobilization [12]. We found that SERCA2 and PLB expression in distal colon smooth muscles were reduced by DSS-colitis, suggesting that SR Ca^{2+} uptake is impaired. This is not due to a generalized decrease in protein expression as smooth muscle myosin heavy chain expression was unchanged. These findings suggest that reduced levels of SERCA2 and PLB contribute to the altered caffeine-evoked Ca^{2+} mobilization of colon smooth muscle cells observed in DSS-colitis. The findings that intracellular Ca^{2+} waves persist in the presence of caffeine suggest that the intrinsic regulation of smooth muscle intracellular Ca^{2+} homeostasis is altered by DSS-colitis, and thus could contribute to the altered contractile responses of colonic smooth muscles. These findings also suggest that down-regulation of SERCA2 and PLB expression, and disruptions in intracellular Ca^{2+} mobilization occur independently of the manner in which colitis is induced and may be a common defect contributing to impaired contractility.

Similar to the frequencies, the amplitudes and AUCs of Ca^{2+} waves were increased, but not significantly, in colon smooth muscles from DSS-colitis mice. Autonomous CaMKII activity in distal colon smooth muscles from DSS-colitis mice was increased, suggesting that the increased amplitudes of Ca^{2+} waves activated CaMKII [37;38]. Unexpectedly, total CaMKII activity was also increased. Elevated Ca^{2+} levels typically do not change total CaMKII activity because it is dependent on the amount of CaMKII [25]. Changes in total CaMKII activity usually indicate changes in CaMKII expression [25]. Western blot analysis confirmed that γCaMKII expression was increased in colon smooth muscles from mice with DSS-colitis. Murine colon smooth muscle cells express the δ and γ CaMKII isoforms [19]. CaMKII activation by $\text{TNF-}\alpha$ has been reported [39] [40], but there are no reports of $\text{TNF-}\alpha$ affecting CaMKII protein expression. However, the murine γCaMKII promoter contains at least one NF- κB response element sequence (5'-GGGCTGCACC-3'), providing a potential mechanism for the increased γCaMKII expression in distal colon smooth muscles during DSS-colitis.

NF- κB activation contributes to impaired smooth muscle contractility [6;30;41]. Proteolytic degradation of $\text{I}\kappa\text{B}\alpha$ allows NF- κB to translocate into the nucleus and initiate gene transcription [42]. Thus, reduced $\text{I}\kappa\text{B}\alpha$ levels strongly suggest NF- κB activation [43]. Our findings show that cytosolic $\text{I}\kappa\text{B}\alpha$ levels in distal colon smooth muscles from mice with DSS-colitis are significantly reduced on days 3-5 post-DSS treatment. These findings are similar to those of Shi et al., showing that $\text{I}\kappa\text{B}\alpha$ is decreased and NF- κB is activated in

canine colon smooth muscles in response to ethanol-acetic acid colitis [44]. These results suggest that, similar to other experimental models of IBD, DSS colitis results in activation of NF- κ B-dependent gene transcription in distal colon smooth muscles.

NF- κ B-dependent gene expression is regulated, in part, by transcriptional co-activators, including HATs and HDACs [17]. The retention of HDAC4 in the cytoplasm by CaMKII binding and phosphorylation allows its target genes to be de-repressed and transcribed [16]. Although indirect, increased cytosolic HDAC4 levels are an indicator of phosphorylation by CaMKII [27]. The sub-cellular localization of HDAC4 was examined to determine whether the increased CaMKII activity resulted in increased phosphorylation of an endogenous CaMKII substrate that plays a role in regulating NF- κ B activity. HDAC4 protein levels in the cytosolic fraction of distal colon smooth muscles from mice with DSS-colitis were significantly increased, suggesting that increased CaMKII activity resulted in increased HDAC4 phosphorylation and cytosolic localization. Since binding of activated CaMKII to HDAC4 is required to sequester HDAC4 in the cytosol [16;27], we utilized a co-immunoprecipitation approach and found that HDAC4 binding to γ CaMKII is increased in distal colon smooth muscle homogenates from mice with DSS-colitis. These findings further support the conclusion that increased CaMKII activity resulted in increased HDAC4 phosphorylation and cytosolic localization. The finding that cytosolic HDAC4 and binding to CaMKII is increased suggests that NF- κ B-dependent gene expression may be de-repressed in distal colon smooth muscles from mice with DSS-colitis.

Higher regulatory systems (enteric motor and sensory neurons, interstitial cells of Cajal, and hormones) control and coordinate gut motility [45]. However, GI smooth muscle cells are equipped with intrinsic regulatory pathways that can amplify or oppose signaling from these higher regulatory systems. The smooth muscle cells of the gut wall are the final effectors of force development and work; thus it is important to understand the mechanisms which mucosal inflammation impairs colon circular smooth muscle contractility. In summary, we have found that alterations in the activities and expression of a subset of proteins involved in intracellular Ca^{2+} mobilization by colonic mucosal inflammation disrupts distal colon smooth muscle function. The results of the muscle tension and Ca^{2+} imaging studies link impairments in intracellular Ca^{2+} mobilization and contractile responses with changes in SERCA2, PLB, and γ CaMKII protein expression in distal colon smooth muscles from mice with DSS-colitis. $\text{I}\kappa\text{B}\alpha$ levels were reduced in colon smooth muscles from DSS-colitis mice, suggesting that NF- κ B is activated. HDAC4 levels and binding to CaMKII were increased, suggesting that CaMKII activation and HDAC4 phosphorylation may facilitate NF- κ B-dependent gene transcription that contributes to the reduced contractility of colon smooth muscles as a result of mucosal inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

2-APB	2-aminoethoxydiphenyl borate
ACh	acetylcholine
CaM	calmodulin
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
cpm	cycles per minute
DSS	dextran sulfate sodium
GI	gastrointestinal
HAT	histone acetyltransferase
HDAC	histone deacetylase
IP₃R	inositol 1,4,5-trisphosphate receptor
iu	intensity units
PLB	phospholamban
RMP	resting membrane potential
ROI	region of interest
SERCA2	SR Ca ²⁺ -ATPase
SR	sarcoplasmic reticulum
ST maps	spatiotemporal maps
STOCs	spontaneous transient outward currents
TTX	tetrodotoxin

Figure 1. Impaired mechanical responses of distal colon smooth muscles from DSS-treated mice
 A. Representative traces of the spontaneous phasic contractile activity of distal colon smooth muscle strips from control and DSS-treated (day 4) mice. B. Representative traces of the mechanical responses of distal colon smooth muscle strips from control and DSS-treated (day 4) mice to 1 μ M ACh. C. Representative traces of the spontaneous phasic contractile activity of distal colon smooth muscle strips from control and DSS-treated (day 4) mice in the presence of 0.3 μ M TTX.

Figure 2. Altered intracellular Ca^{2+} wave properties in distal colon smooth muscles from DSS-treated mice

Representative Z-compressed images showing the region of interest (ROI) in distal colon smooth muscles from control (A) and DSS-treated mice (day 4) (B) from which intracellular Ca^{2+} waves were recorded and spatio-temporal maps constructed. Representative spatio-temporal maps of intracellular Ca^{2+} waves in distal colon smooth muscles from control and DSS-treated mice (day 4) in the absence (C, D) or presence (E, F) of 1mM caffeine for 10 min. horizontal scale = 2 sec; vertical scale = 10 μm . Bar graphs of the frequencies (G), amplitudes (H), and AUC (I) of intracellular Ca^{2+} waves without and with caffeine. open bars, control; filled bars, day 4 post-DSS treatment; * $P < 0.05$ compared to - caffeine, # $P < 0.05$ compared to control + caffeine.

Figure 3. Effect of caffeine on electrical responses of distal colon smooth muscles from control and DSS-treated mice

A. Representative microelectrode recordings of the hyper-polarization of distal colon smooth muscle membrane potentials from control and DSS-treated mice (day 4) in response to 1mM caffeine. B. Bar graph of the Δ RMP from control and DSS-treated mice in response to 1mM caffeine. * $P < 0.05$.

Figure 4. SERCA2 and PLB expression is decreased in distal colon smooth muscles from DSS-treated mice

Representative western blots of SERCA2 (A), and PLB (C) in distal colon smooth muscles SR fractions from control (Con) and DSS-treated mice on days 1-5 post-DSS-treatment. Each lane contains 50 μ g of protein. Lower panels are immunoblots of smooth muscle myosin heavy chain (5 μ g protein per lane). Densitometry of SERCA2 (B), and PLB (D) western blots from control (Con) and DSS-treated mice at days 1-5 post-DSS treatment. Control intensity values from the densitometry analyses were normalized to 1. (n=5 western blots from 5 different sets of control and DSS-treated mice, * $P < 0.05$ compared to control).

Figure 5. CaMKII activity and expression are increased in distal colon smooth muscles from DSS-treated mice

Autonomous (A) and total (B) CaMKII activity in distal colon smooth muscle homogenates from control (Con) and DSS-treated mice at days 1-5 post-DSS treatment (n=7, $*P < 0.05$ compared to control). Representative western blot of γ CaMKII (C) in distal colon smooth muscle cytosolic fractions from control and DSS-treated mice on days 1-5 post-DSS treatment. 50 μ g protein per lane. Densitometry of γ CaMKII western blots from control (Con) and DSS-treated mice on days 1-5 post-DSS treatment (D). Control intensity values from the densitometry analyses were normalized to 1. (n=5 western blots from 5 different sets of control and DSS-treated mice, $*P < 0.05$ compared to control).

Figure 6. IκBα levels are decreased in distal colon smooth muscles from DSS-treated mice

A. Representative western blot of IκBα in distal colon smooth muscle cytosolic fractions from control and DSS-treated mice on days 1-5 post-DSS treatment. 50 μg protein per lane.

B. Densitometry of IκBα western blots from control (Con) and DSS-treated mice on days 1-5 post-DSS treatment. Control intensity values from the densitometry analyses were normalized to 1. (n=4 western blots from 4 different sets of control and DSS-treated mice, **P* < 0.05 compared to control).

Figure 7. Cytosolic HDAC4 levels and HDAC4- γ CaMKII interaction are increased in distal colon smooth muscles from DSS-treated mice

A. Representative western blot of HDAC4 in distal colon smooth muscle cytosolic fractions from control and DSS-treated mice on days 1-5 post-DSS treatment. 50 μ g protein per lane. B. Densitometry of HDAC4 western blots from control (Con) and DSS-treated mice on days 1-5 post-DSS treatment. Control intensity values from the densitometry analyses were normalized to 1. (n=4 western blots from 4 different sets of control and DSS-treated mice, $*P < 0.05$ compared to control). C. Distal colon smooth muscle cytosolic fractions from control and DSS-treated mice on day 3 post-DSS treatment were immunoprecipitated with anti- γ CaMKII antibodies and then immunoblotted with anti-HDAC4 antibodies. Representative western blot of four experiments.