Divergent changes to muscarinic and serotonergic signalling following colitis

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Gut 2005;54:1699–1706. doi: 10.1136/gut.2005.066563

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Revised version received
13 June 2005

Accepted for publication
21 June 2005

Published online first
13 July 2005

Intestinal inflammation impairs agonist induced contraction of smooth muscle (see Moreels and colleagues,¹ Cao and colleagues,² and Shi and Sarna³, and reviewed in Sarna⁴ and Collins⁵) but the cellular basis for this remains to be fully elucidated. Furthermore, the diversity of neurotransmitters and other contractile agonists present within the inflamed intestine adds further complexity to the understanding of altered contractility. However, much of the current literature has focused on the contractile response to acetylcholine (ACh) as the primary neurotransmitter of the gut, and few studies have evaluated contractility in response to other excitatory agonists such as serotonin (5-hydroxytryptamine; 5-HT). Recently, we showed that decreased contractility in trinitrobenzene sulphonic acid (TNBS) induced rat colitis includes a significant component of change at the level of the circular smooth muscle cell (CSMC).⁶ During peak colitis on day 4 (D4), CSMC isolated from the inflamed mid-descending colon showed an attenuated contractile response to both carbachol (CCh) and 5-HT. After resolution of overt inflammation by day 36 (D36), the impaired CCh induced contractile response returned to control values. However, the response to 5-HT remained impaired, showing that colitis differentially affects these receptor systems.⁶

ACh induces contraction of CSMC by interacting with muscarinic receptors 2 and 3 (M_2R and M_3R).⁷ Signalling through both of these receptors has the capacity to increase the intracellular calcium concentration where M_3R signalling increases inositol 1,4,5-trisphosphate (IP_3) and M_2R inactivates adenylyl cyclase.^{8,9} In a parallel system, 5-HT interacts with its specific receptors where intestinal smooth muscle cells express the 5-HT_{2A} receptor (5-HT_{2AR}) and 5-HT_{4R} subtypes,^{10–12} while fundic smooth muscle expresses the

Background: The altered motility of the inflamed intestine derives in part from changes to the contractility of the intestinal smooth muscle cell. While modifications to the muscarinic receptor system are identified, changes to 5-hydroxytryptamine (5-HT; serotonin) receptors that also mediate contraction are less well studied.

Methods: In the trinitrobenzene sulphonic acid model of rat colitis, we used receptor antagonists to identify changes in receptor utilisation that accompany the selective reversal of the impaired contractile response to acetylcholine (ACh) and 5-HT during colitis (day 4 (D4)) and following resolution of inflammation (day 36 (D36)).

Results: In isolated circular smooth muscle cells, challenged with ACh, the muscarinic 3 receptor (M_3R) antagonists 4-DAMP and pF-HSD each showed a 50% decrease in antagonism on D4 while the M_2R antagonist methoctramine more than doubled its potency, showing a decreased role of M_3R and an increased role of M_2R , respectively. These changes were fully reversed by D36. In contrast, the 5-HT₂ receptor (5-HT_{2R}) antagonist ketanserin was sharply decreased in effectiveness on D4, with a further decrease by D36, when the contribution of 5-HT_{2AR} was only 22% of control. There were no changes in response to the 5-HT_{4R} antagonist SDZ-205-557 at any time. Western blotting identified decreased expression of 5-HT_{2AR} on D36 versus controls, further supporting the conclusion that the persistence of the impaired response to 5-HT was due to decreased expression of the excitatory 5-HT_{2AR}.

Conclusions: Thus the lasting decrease in receptor expression and resulting impairment of the contractile response will compromise the capacity for an appropriate response to 5-HT, which may contribute to the intestinal dysfunction seen in post-enteritis syndromes.

5-HT_{2B}R subtype (for example, see Cox and Cohen¹³). In contrast with the muscarinic signalling system where both receptors are positively linked to contraction, 5-HT receptors diverge in their signalling pathways: the excitatory 5-HT_{2AR}, like M_3R , raises Ca^{2+}_i via increased IP_3 production^{10–14} while 5-HT_{4R} is positively coupled to adenylyl cyclase and hence inhibits contraction by antagonising calcium mobilisation.^{10–14–15}

The essential role of muscarinic receptors in cholinergic contraction of CSMC has been well established but the physiological role of the serotonin receptors on these cells remains poorly understood, despite their ability to mediate a rapid and extensive contractile response equivalent to that induced by acetylcholine (for example, see Wells and Blennerhassett⁶). In intact tissue as well as in isolated cell preparations, exogenous 5-HT acts directly to contract intestinal smooth muscle from humans,¹⁰ and other species (for example, porcine¹⁶), further supporting a potentially important role in motility.

The origin of the 5-HT that might act on these receptors in vivo is less clear. While the presence of 5-HT containing neuronal cell bodies within the myenteric plexus of several species has been long recognised,¹⁷ immunocytochemical studies have only recently shown 5-HT containing varicose axons within the circular smooth muscle of the rat^{18–19} and

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); 5-HT_{2AR}, serotonin receptor #; ACh, acetylcholine; BSA, bovine serum albumin; CCh, carbachol; CSMC, circular smooth muscle cell; D0, D4, D36, days post initiation of colitis; DMSO, dimethylsulphoxide; HPSS, HEPES physiological salt solution; IBS, irritable bowel syndrome; IP_3 , inositol 1,4,5 trisphosphate; MPO, myeloperoxidase; M_2R , muscarinic receptor #; TBS-T, Tris buffered saline with 0.25% Triton X-100; TNBS, trinitrobenzene sulphonic acid

rabbit intestine.²⁰ Evidence supporting neurally mediated contraction of intestinal smooth muscle via 5-HT release is therefore limited, and non-neuronal sources of 5-HT may also be important. Immune cells, such as mast cells, and platelets contain 5-HT and their activation in inflammation could directly influence CSMC contraction.²¹ Furthermore, increased availability of 5-HT due to decreased inactivation occurs in the inflamed mucosa,²² raising the possibility of indirect action via spillover on the underlying muscle layers.

Inflammation also affects smooth muscle contraction through alterations in the cholinergic receptor system. Under control conditions, ACh induced contraction is mediated solely through the M₃R but during inflammation the M₂R pathway becomes functionally active.^{23–25} If a similar phenotypic change occurred in the 5-HT receptor system, as suggested by results to date,⁶ profound changes to smooth muscle function might result during inflammation which, if not reversed, might also influence motility in the recovered state. No studies have examined smooth muscle cell receptor usage following resolution of inflammation.

We studied this in the rat colon, hypothesising that alterations to the 5-HT and ACh receptors systems might contribute to altered CSMC contraction that occurs during inflammation induced with TNBS. Our goals were to: (1) examine the impact of colitis on ACh and 5-HT induced contraction using freshly isolated single CSMC over a full time course of inflammation (onset to overt recovery); (2) examine changes to muscarinic and serotonergic receptor expression following resolution of colitis; and (3) compare modifications to the 5-HT and ACh receptor systems.

MATERIALS AND METHODS

TNBS induced inflammation

All experiments were performed on adult male Sprague-Dawley rats (Charles River, Quebec, Canada). Animals were housed in pairs in microfilter isolated cages with free access to food and water for at least five days before initiation of experimental procedures. For experimental colitis, rats were lightly anaesthetised with halothane, and 500 µl of 200 mM TNBS (Sigma, St Louis, Missouri, USA) dissolved in 50% ethanol was introduced into the colon 8 cm proximal to the anus using a PE-50 catheter as previously described.^{6, 26} All experimental procedures were approved by the local university animal care committee.

Histology

Animals were sacrificed at various times after initiation of colitis, and the inflamed region of the mid descending colon was removed, followed by routine processing for paraffin sectioning. Histological sections were stained with haematoxylin-eosin for examination by a observer blinded to the experimental condition, according to the procedures described.⁶ In addition, myeloperoxidase (MPO) activity was assessed as previously described.⁶ Briefly, snap frozen intestinal segments were placed into a solution (1 ml/50 mg tissue) of hexadecyltrimethyl-ammonium bromide (0.5% wt/vol) in potassium phosphate buffer (50 mM, pH 6.0). Samples were homogenised on ice for 30 seconds, centrifuged for 30 minutes at 10 000 rpm at 4°C, and then incubated at 60°C for two hours. After incubation, 100 µl of supernatant were added to 2.9 ml of 0.00005% H₂O₂ in an o-dianisidine dihydrochloride (Sigma, St Louis, Missouri, USA) solution in a cuvette, and absorbance was measured at 460 nm. Results were expressed as units of MPO activity per 100 mg of tissue (wet weight), where 1 unit of MPO activity is the amount of enzyme required to split 1 µmol H₂O₂/min at 25°C.

Isolation of circular smooth muscle cells

Isolation of colonic CSMC was carried out as previously described.^{6, 27} Briefly, the mid-descending colon was removed and placed in fresh Krebs solution, and the mucosa and submucosa were removed by sharp dissection. Strips of circular smooth muscle were removed and placed into HEPES physiological salt solution (HPSS) digestion solution incubated at 5°C for two hours, at room temperature for 20 minutes, and finally at 31°C for 10 minutes. The solution containing the circular smooth muscle was then poured over a 200 µm nylon mesh filter and thoroughly rinsed with HPSS isolation solution to remove excess enzyme solution, resuspended in HPSS isolation solution and gently triturated to produce a suspension of individual CSMC.

Morphological evaluation of isolated CSMC

Phase contrast microscopy (Olympus IMT-2) was used to identify cells that were phase bright with membranes free of physical distortions and blebbing. Such cells were determined to be 100% viable by exclusion of trypan blue staining (data not shown). Overall, ≥90% of the CSMC population demonstrated these characteristics.

Assessment of individual cellular contractility

The effects of inflammation on agonist induced contractility of individual CSMC were directly examined, as previously described.⁶ Briefly, aliquots of freshly isolated CSMC were placed in a 35 mm dish with inset glass coverslips, allowed to settle for 10 minutes, and viewed using an inverted phase contrast microscope (Olympus IMT-2) connected to a closed circuit video camera. Individual CSMC were directly exposed to either ACh or 5-HT (1 µM in Krebs solution) by pressure application via a puffer pipette (500 ms application at 10 psi through a 1 MΩ resistance tip) which was micromanipulated to within 200 µm of the cell. CSMC were continuously perfused with Krebs solution at a rate of 2 ml/min. Images were captured at 250 ms intervals, allowing identification of the resting length and maximal contractility of the cells isolated on days 0, 4, and 36 (D0, D4, and D36). Length measurements were obtained using an image analysis program (ImagePro 5.0; Media Cybernetics, Silver Spring, Maryland, USA). Negative controls included the pressure application of Krebs solution via puffer pipettes, which failed to induce contraction (data not shown).

Receptor antagonism

The effects of specific receptor antagonists were determined on freshly isolated colonic CSMC. Briefly, CSMC were plated and control responses to ACh or 5-HT were determined for each dish of cells, as described above. This per cent contraction was then taken as the maximal response for each experimental condition (D0, D4, and D36), thus normalising for the inflammation induced changes to maximal contraction. Receptor antagonists were added and incubated for 10 minutes and the above procedure was repeated on another population of cells in the same dish. Any changes in the maximal contraction were then expressed as a per cent of the appropriate control response. This was repeated for each antagonist examined. Concentrations of antagonists used were the maximal concentrations shown to be selectively effective.^{10, 23, 25, 28–32} In all cases, application of antagonist or dimethylsulphoxide (DMSO) had no effect on resting cell length, and DMSO alone did not alter agonist induced contractility (data not shown).

Immunocytochemistry

To address a mechanism for the inflammation induced attenuation in M₃R signalling, the presence of the M₃R was determined using immunocytochemistry on formalin fixed

paraffin embedded sections. Following antigen retrieval using a target retrieval solution (Dako, Mississauga, Ontario, Canada), sections were blocked in 1% goat serum and incubated with anti-M₃R antibody (1:100; Santa Cruz, Santa Cruz, California, USA) overnight at 4°C, followed by a one hour incubation with goat antirabbit Alexa 555 (1:1000; Molecular Probes, Eugene, Oregon, USA). Labelling was visualised with an Olympus BX-51 microscope and images were digitally captured (ImagePro 5.0; Media Cybernetics).

Immunocytochemistry for 5-HT_{2A}R was performed as previously described.³³ Briefly, the mid-descending colon was formalin fixed and embedded for cryosectioning. Floating sections (40 µm) were permeabilised and blocked for one hour (Tris buffered saline containing 0.25% Triton X-100 (TBS-T) with 2% bovine serum albumin (BSA)). Sections were incubated with anti-5-HT_{2A}R antibody (2 µg/ml in TBS-T with 2% BSA for 48 hours at 4°C; BD Pharmingen, San Diego, California, USA), followed by a one hour incubation with goat antimouse Alexa 555 (1:1000; Molecular Probes). Nuclear localisation was obtained by staining with Hoechst 33342 (1:5000). Labelling was visualised as above.

Immunocytochemistry for 5-HT_{2A}R was also performed on freshly isolated CSMC. Briefly, cell suspensions were formalin fixed, washed, and blocked in TBS-T containing 2% BSA for one hour. Cells were incubated with 2 µg/ml anti-5-HT_{2A}R antibody for two hours at room temperature, followed by a one hour incubation with goat antimouse Alexa 488 (1:1000; Molecular Probes), with nuclear staining using propidium iodide (10⁻⁹M). After each step, suspension of CSMC was centrifuged at 1000 *g* for four minutes and resuspended in the appropriate solution. Antibody labelling was visualised using confocal microscopy with optical sectioning and 3-dimensional reconstruction using Fluoview software.

Western blotting

Studies using the selective 5-HT₂ antagonist demonstrated a sustained decrease in 5-HT₂ signalling. Therefore, 5-HT_{2A}R expression was evaluated using western blot analysis of freshly isolated smooth muscle tissue, as described previously.³⁴ After protein determination of tissue lysates, samples containing equal amounts of protein were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After transfer, membranes were incubated with either anti-5-HT_{2A}R antibody (1 µg/ml for 48 hours) or anti-β-actin antibody (1:1000 for 24 hours) in TBS-T with 5% milk at 4°C, washed in TBS-T, incubated for one hour in horseradish peroxidase conjugated goat antimouse (1:10000; Pierce, Rockford, Illinois, USA), and visualised by enhanced chemiluminescence following the manufacturer's instructions. X ray films were scanned and band intensity was determined using Image Pro software. The 5-HT_{2A}R values were then normalised to β-actin expression and expressed relative to control.

Chemicals and solutions

The composition of solutions were as follows: Krebs solution (in mM: NaHCO₃ 25; NaCl 118; KCl 4.7; NaH₂PO₄ 1; MgSO₄ 1.2; glucose 11; CaCl₂ 2.5; bubbled with 95%O₂/5%CO₂); HPSS digestion solution (in mM: NaCl 125; glucose 10; Na-HEPES 10; MgCl₂ 1; KCl 4; CaCl₂ 1; EDTA 0.25; taurine 10; pH 7.2) with papain (0.5 mg/ml), BSA (1 mg/ml), DL-dithiothreitol (1 µM), and collagenase type-F (0.5 mg/ml); HPSS isolation solution (in mM: NaCl 112.5; KCl 5.5; KH₂PO₄ 2; Na-HEPES 24; CaCl₂ 1.9; MgCl₂ 0.6; glucose 8; BME amino acids 40 µl/ml; soybean trypsin inhibitor 0.1 mg/ml, pH 7.4). Receptor antagonists used were: M₂R, 1 µM methoctramine; M₃R, 1 µM 4-DAMP; M₃R, 1 µM pF-HSD;

Figure 1 Inflammation reversibly impairs acetylcholine (ACh) induced contraction of circular smooth muscle cells (CSMC) from the rat colon. CSMC were enzymatically isolated on day 0 (D0, control), day 4 (D4), and day 36 (D36) post colitis, and contraction assessed under direct microscopic visualisation at 37°C. (A–C) Micrographs showing contraction of an individual D0 CSMC following application of 1 µM ACh by micropipette, seen on the right side of each image (arrowhead). (A) Control; (B) +750 ms; and (C) +3500 ms after ACh application. Scale bar 100 µm. (D) Time course of mean per cent contraction of cells from D0, D4, and D36 animals in response to ACh (*n* = 3 animals per category at 10 cells/animal). Arrows indicate per cent contraction of the cell illustrated in (A–C). Per cent contraction for D4 was significantly less than that for D0 and D36 at all time points ≥ 500 ms (*p* ≤ 0.05).

5-HT_{2A}R, 1 µM ketanserin; and 5-HT₄R, 1 µM SDZ-205-557. Except where mentioned, all chemicals were purchased from Sigma.

Data analysis

All data are expressed as mean (SEM). Statistical analysis was performed using one way analysis of variance (ANOVA) with the Student Newman-Keuls multiple comparison test. For contractility experiments, data were fitted using a standard curve fit equation with weight on SEM. A *p* value of ≤ 0.05 was considered statistically significant.

RESULTS

TNBS induced inflammation of the mid-descending rat colon

Administration of TNBS caused colitis with the presence of an overtly inflamed colon by D4, as previously described.^{6, 26} Involved areas were identified by a severely damaged mucosa, oedema, and adhesions. By D36, all overt signs of inflammation had resolved but previously involved areas could be identified by bowel wall thickening (a result of inflammation induced hypertrophy and hyperplasia).^{35, 36} In all cases, inflammation was verified using the independent

Figure 2 Inflammation alters utilisation of muscarinic receptors in acetylcholine (ACh) induced contraction of colonic circular smooth muscle cells isolated from rats on day 0 (D0, control), day 4 (D4), and day 36 (D36) post colitis. ACh was applied in the presence of the selective muscarinic M_2 (methoctramine) and M_3 (4-DAMP or F-HSD) receptor antagonists. Data are presented as per cent inhibition of maximal contraction under each condition (that is, per cent of the contraction of cells receiving no antagonist). Each population of cells served as their own control for maximal contraction before treatment, thus normalising for the differences in contraction that occur during inflammation. During inflammation (D4), the M_3 antagonists pF-HSD (A) and 4-DAMP (B) became significantly less effective at blocking contraction but this decrease was reversed following resolution of the inflammation (D36). In contrast, the M_2 antagonist methoctramine (C) showed increased effectiveness in blocking contraction during inflammation, which was also reversed following colitis. $n = 4-6$ animals, with 10 cells/animal. * $p \leq 0.05$ versus D0.

criteria of weight loss, histological evaluation of transmural inflammation, and MPO analysis.

We have previously described the time course of TNBS induced inflammation using both microscopic evaluation and MPO.⁶ In this study, a similar time course was observed with maximal inflammation present by D4 and full resolution by D36 (data not shown). In addition, our MPO results were similar to those obtained in previous studies (data not shown).^{6, 37, 38}

ACh induced contraction during inflammation

To establish whether inflammation modified cholinergic induced contractility, CSMC were exposed to ACh under microscopic visualisation. ACh application to control (D0) CSMC resulted in maximal contraction of 26.3 (0.4)% ($n = 3$), occurring approximately three seconds after the onset of contraction (fig 1). By D4, the response to ACh showed significant attenuation in maximal per cent contraction ($p \leq 0.05$). In addition, there was a significant increase in the time required for half maximal contraction, from 758.3 (57.3) ms in D0 CSMC to 1046.7 (61.2) ms on D4 ($p \leq 0.05$). However, these changes were completely reversed on resolution of inflammation (D36), with contractility of CSMC from D36 animals indistinguishable from D0 responses (fig 1D).

Changes to muscarinic receptors during inflammation

Previous studies demonstrated that inflammation caused alterations to the use of muscarinic receptors at the level of the smooth muscle cell.^{3, 23, 25} However, it is not known whether there was reversal of these modifications following recovery from inflammation. We explored this pharmacologically using selective muscarinic receptor antagonists at concentrations previously shown to be maximally effective in intestinal smooth muscle while having minimal non-specific antagonism of other muscarinic receptor subtypes (for example, see Shi and Sarna²³ and Sohn and colleagues²⁵). Data were expressed as per cent of inhibition of the ACh response. Pretreatment of isolated CSMC with the selective M_3 R antagonists pF-HSD or 4-DAMP caused a 51 (7)% and 63 (9)% reduction in the contraction of individual control CSMC, respectively (fig 2A, B). Furthermore, application of the selective M_2 R antagonist methoctramine (fig 2C) had a small but consistent effect on maximal contraction, causing a 12 (4)% reduction ($p < 0.05$ v control) that was substantially less than the effect of M_3 R antagonists. This establishes the characteristic proportions of muscarinic receptor involvement in response to cholinergic stimulation of D0 CSMC.

In CSMC isolated on D4 of colitis, the effectiveness of the M_3 R antagonists in blocking ACh induced contraction was significantly reduced ($p \leq 0.05$). 4-DAMP and pF-HSD blocked only 26 (9)% and 24 (5)% of ACh induced contractions, respectively, approximately 50% of their D0 effect. In contrast, the effectiveness of methoctramine was increased significantly, and it antagonised ACh induced contraction by 29 (6)%, more than double that measured at D0 ($p \leq 0.05$). This shows that colitis induces a shift in the pattern of utilisation of muscarinic receptors, with decreased reliance on M_3 R and increased utilisation of M_2 R.

On resolution of the inflammation, there was complete reversal of the changes to muscarinic receptor utilisation. The effectiveness of methoctramine was reduced to D0 levels as it blocked 17 (6)% of ACh induced contraction. Furthermore, the effect of 4-DAMP and pF-HSD returned to D0 values, blocking 56 (9)% and 57 (10)% of the contraction, respectively. Application of the non-specific muscarinic antagonist atropine abolished ACh induced contraction at all time points (data not shown), indicating that all contractile responses were due to cholinergic signalling.

Due to the large decrease in M_3 R utilisation during colitis, we examined expression of M_3 R on CSMC. Immunocytochemistry was used to visually assess the presence and distribution of the receptor at the cellular level. In D0 tissue, M_3 R labelling was distributed throughout the circular and longitudinal smooth muscle layers, with a uniform punctate staining pattern (fig 3A). By D4, the appearance of M_3 R labelling was characteristically altered, with diminished membrane localisation and the appearance of strong perinuclear labelling (fig 3B). However, by D36, the appearance of M_3 R labelling was again similar to control,

Figure 3 Alterations in expression of muscarinic M_3 receptors (M_3R) in colitis. Photomicrographs of immunofluorescence detecting M_3R in sections of normal and inflamed colonic circular smooth muscle from (A) control (day 0), (B) day 4, and (C) day 36 tissue. Day 0 and day 36 tissue demonstrated a similar diffuse staining pattern that was membrane associated. In contrast, M_3R localisation on day 4 smooth muscle was largely perinuclear (arrows), suggesting internalisation of the M_3R . Scale bar 20 μ m.

with a uniform distribution of receptor staining (fig 3C). Negative controls (primary antibody omission and mismatched secondary antibody) failed to produce significant labelling (data not shown).

5-HT induced contraction during inflammation

Direct exposure of D0 CSMC to 5-HT via pressure application caused maximal contraction of 24.8 (0.9)% ($n = 3$), occurring

Figure 4 Colitis causes lasting impairment to 5-hydroxytryptamine (serotonin; 5-HT) induced contraction of isolated circular smooth muscle cells (CSMC). Time course of contraction of CSMC isolated from day 4 (D4) or day 36 (D36) animals showed a significant decrease in 5-HT induced response by D4 and a further significant decrease by D36 relative to control (D0). Data points represent the mean per cent contraction ($n = 3$ animals per condition at 10 cells/animal). Per cent contraction for D4 and D36 was significantly less than for D0 at all time points ≥ 500 ms. Per cent contraction for D36 was significantly less than that for D4 at all time points ≥ 2000 ms ($p \leq 0.05$).

approximately three seconds after the onset of response (fig 4). By D4, there was a significant modification of 5-HT induced contraction ($p \leq 0.05$), with both decreased sensitivity and attenuation in the maximal per cent contraction (18.8 (0.3)%). In addition, there was a significant increase in the time required for half maximal contraction, from 670.0 (20.8) to 963.3 (62.3) ms ($p \leq 0.05$). By D36, the 5-HT induced contractile response remained altered from control, and in fact was further suppressed beyond that seen in CSMC from D4 animals, with maximal contraction decreased to 14.9 (0.8)%.

Changes to 5-HT receptors during inflammation

Immunocytochemistry was used to visualise the presence of the 5-HT_{2A}R in the circular smooth muscle layer of the rat colon. In cryosections, 5-HT_{2A}R labelling was present at the membrane junctions between adjacent CSMC (fig 5A). To further study this cellular localisation, immunocytochemistry was repeated on isolated CSMC. Using confocal microscopy, positive staining for 5-HT_{2A}R was observed in linear arrays along the surface of the cell (fig 5B). In both cases, the absence of primary antibody or the use of a mismatched secondary failed to produce significant labelling.

As inflammation resulted in altered responses to activation of muscarinic receptors, we tested whether similar changes to the utilisation of serotonin receptors in the contractile response to 5-HT were also present. This was achieved by preincubation with the selective 5-HT₂ and 5-HT₄ receptor antagonists (ketanserin and SDZ-205-557, respectively) at concentrations shown to be specifically and maximally effective.^{10 39–42} All data were expressed as a per cent of inhibition of the 5-HT response. In D0 CSMC, application of the 5-HT₂R antagonist ketanserin (1 μ M) resulted in a 69 (4)% decrease in the response to 5-HT (fig 6A). This inhibition was much less effective by D4 where ketanserin inhibited the 5-HT response by only 33 (6)%.

Figure 5 The excitatory 5-hydroxytryptamine receptor 2A (serotonin; 5-HT_{2A}R) is expressed in rat colonic smooth muscle. (A) Immunocytochemistry of a section of rat mid-descending colon showing localisation of 5-HT_{2A}R labelling (red) to smooth muscle cell junctions, with nuclear staining (blue) using Hoechst 33342. (B) Confocal microscopy showing 5-HT_{2A}R (green) on the surface of an isolated circular smooth muscle cell, with nuclear staining using propidium iodide (red). Staining for the 5-HT_{2A}R was localised in linear arrays on the cell surface. Scale bars 25 μ m (A) and 10 μ m (B).

The diminished actions of ketanserin did not reverse with resolution of colitis. By D36, ketanserin showed very little effect, blocking only 15 (5)% of 5-HT induced contraction. As the impaired 5-HT induced contraction might represent an increase in expression of the inhibitory 5-HT₄R, we examined contraction in the presence of a selective 5-HT₄R antagonist SDZ-205-557. This had no effect on contraction at any time points (fig 6B), indicating no apparent involvement of this receptor subtype in the altered response to 5-HT.

The reduced effectiveness of the 5-HT_{2A}R antagonist after the onset of colitis suggested that inflammation might cause the decreased expression of this receptor. This was studied by western blot analysis on tissue from control (D0), D4, and D36 animals using a specific anti-5-HT_{2A}R antibody. Figure 7 illustrates the typical outcome observed with western blotting, where predominant bands were identified at approximately 55 kDa, similar to that reported previously.⁴³ The intensity of the signal was reduced by D4, and remained suppressed on D36. Image analysis of multiple independent experiments demonstrated an 80% reduction in protein expression by D4, which became further reduced to only 32% of control on D36 post-TNBS (fig 7). Taken together, these results suggest that inflammation resulted in a lasting decrease in expression of the excitatory 5-HT_{2A}R.

Figure 6 Inflammation alters the utilisation of the 5-hydroxytryptamine (serotonin; 5-HT) receptor in contraction of circular smooth muscle cells. 5-HT was applied in the presence of the 5-HT receptor 2A (5-HT_{2A}R) antagonist ketanserin (A) or the 5-HT₄R antagonist SDZ-205-557 (B), with data presented as per cent inhibition of contraction relative to contraction of cells receiving no antagonist. Each population of cells served as their own controls for maximal contraction, thus normalising for the differences in contraction during inflammation. Ketanserin was less effective at blocking contraction during inflammation and there was no recovery of this impairment following resolution of the colitis. No changes were observed in the effectiveness of SDZ-205-557, showing no alteration in the contribution of 5-HT₄R to contraction. $n = 3$ animals per time point, with 10 cells/animal per condition; * $p \leq 0.05$ versus day 0 (D0). D4, D36, days 4 and 36, respectively.

DISCUSSION

The origins of the altered motility seen in the inflamed intestine include direct modification of the functional nature of the individual smooth muscle cell. Here we demonstrated that inflammation caused substantial, but divergent, changes to two receptor systems that are involved in initiation of smooth muscle contraction. Specifically, we showed inflammation induced dysfunction of both cholinergic and serotonergic pathways, associated with functional modifications at the receptor level. While impaired activation of contraction was characteristic of both systems in CSMC from inflamed tissue, there was selective reversal of these changes following resolution of colitis. This is novel evidence for the existence of separate regulatory pathways for the control of expression and function of these receptors, which become apparent during and after inflammation.

It has been well established that intestinal CSMC express the muscarinic receptors M₂R and M₃R in a ratio of approximately 4:1, respectively.^{8,9,44} Here we demonstrated that the ACh induced contraction of rat colonic CSMC was mediated predominantly by M₃R as selective M₃R antagonists blocked most of the response. However, there was a minor M₂R mediated component, as application of methocarbamol caused a 12% reduction in ACh induced cell

Figure 7 Western blot analysis showing decreased expression of 5-hydroxytryptamine receptor 2A (serotonin; 5-HT_{2A}R) during colitis. Inset, representative blots for 5-HT_{2A}R in control (day 0, D0), day 4 (D4), and day 36 (D36) animals identifying the presence of the receptor in colonic smooth muscle. Graph shows outcome of image analysis of immunoblots for 5-HT_{2A}R, with integrated optical density (IOD) normalised against β-actin and expressed relative to control. By D4, there was a significant decrease in the amount of 5-HT_{2A}R present in smooth muscle, and this remained decreased on D36. *n* = 5–6 experiments using *n* = 3–4 rats; **p* ≤ 0.05 versus D0.

shortening. While similar methoctramine induced decreases in contraction were observed by Sohn and colleagues,²⁵ these studies were performed on smooth muscle cells isolated from the cat lower oesophageal sphincter. Nevertheless, the decreased contraction in the presence of methoctramine was modest compared with the effects of the M₃R antagonists 4-DAMP and pF-HSD. Therefore, our data showed that ACh induced contraction was predominantly mediated by M₃R under control conditions, which is consistent with findings in other systems.

Acute inflammation has been shown to cause a functional switch in the roles of the muscarinic receptors.^{3, 23, 24} In those studies, the inflammation induced decrease in CSMC contraction paralleled modulation of muscarinic receptor utilisation. We found a parallel decrease in the functional utilisation of the M₃R in the inflamed colon, as the selective antagonists 4-DAMP and pF-HSD became less effective in blocking contraction. As M₃R utilisation decreased, there was a substantial increase in the dependency on M₂R in ACh mediated contraction, as methoctramine displayed increased effectiveness on D4 of colitis.

Proposing that this may also reflect altered expression, we used immunocytochemistry to visualise modifications in M₃R localisation. This suggested a shift in distribution away from the surface membrane towards an intracellular perinuclear localisation, compatible with the alterations seen in cellular contractility. In contrast, the predominant muscarinic receptor expressed on the CSMC is M₂R, even under control conditions. Therefore, the subtle changes in M₂R use are not likely due to increased receptor expression, and may originate from intracellular changes that lead to unmasking of this receptor.

As inflammation caused functional changes in muscarinic receptor expression, we hypothesised that similar changes might occur for 5-HT, as another dual receptor mediated system in CSMC which expresses the serotonin receptors 5-HT_{2A}R and 5-HT₄R.^{10, 45} Therefore, we examined whether colitis changed the relative roles of these receptors in cellular contractility. Using the selective 5-HT receptor antagonists, ketanserin and SDZ-205-557, we showed that 5-HT_{2A}R

mediated effects remained suppressed following resolution of inflammation, but that no changes occurred in the 5-HT₄R pathway. Further analysis using western blotting showed that a decrease in 5-HT_{2A}R protein expression underlies this, and is at least partly responsible for the impaired serotonin induced contractile response seen following colitis.

In addition to 5-HT_{2A}R in intestinal smooth muscle,^{10, 12, 16} some studies suggest the presence of other excitatory 5-HT receptors, such as the 5-HT_{2B}R that is also inhibited by ketanserin.⁴⁶ This, and the presence of other excitatory 5-HT receptors such as the 5-HT₁R identified on vascular smooth muscle cells,⁴⁷ may help to explain residual ketanserin insensitive contraction. While beyond the scope of this paper to characterise all of the 5-HT receptors present in our tissue, our data demonstrated a lasting physical decrease in the primary excitatory serotonergic receptor, 5-HT_{2A}R. This contrasts with the pharmacological and immunocytochemical evidence showing complete recovery from decreased M₃R utilisation in colitis. Overall, evidence from this animal model of colitis suggests that modulation of receptors responsible for excitatory signalling to smooth muscle may constitute an important aspect of the changes present in the inflamed intestine.

Furthermore, the continued presence of some of these changes after colitis shows that the capacity for an appropriate response to 5-HT in either physiological or pathophysiological circumstances becomes altered, which may be significant in understanding the nature of the irritable bowel syndrome (IBS) that may develop following an episode of intestinal inflammation. For example, recent work shows that the 5-HT₄R agonist tegaserod that is used in IBS therapy also antagonises both 5-HT_{2A}R and 5-HT_{2B}R, at concentrations similar to those that activate 5-HT₄R.⁴⁸ While still unclear how this additional role is involved in amelioration of impaired motility, it is interesting to speculate that attenuation of 5HT_{2A}R, seen to occur in colitis and to persist in the recovered animal, might contribute to the appearance of a receptor profile that favours the beneficial actions of 5HT₄R agonists, such as tegaserod. Further research is needed to determine the changes to 5HT₂R expression in human disease, and in particular in post-inflammatory IBS.

ACKNOWLEDGEMENTS

The authors thank Dr S Lourenssen for help in preparation of this manuscript and Kurtis Miller for technical assistance. We thank the Canadian Digestive Health Foundation and CIHR for scholarship support (RWW).

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Conflict of interest: None declared.

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