Colonic Inflammation Increases the Contribution of Muscarinic M2 Receptors to Carbachol-Induced Contraction of the Rat Colon

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Abstract

Objectives: Carbachol-induced contraction of the rat colon is impaired in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis. The main objective of this study was to examine the effect of colitis on the expression and function of muscarinic (M) receptor subtypes in the rat colon. Materials and Methods: Rats (n = 80) were treated with TNBS and used 5 days later for measurement of contractility, myeloperoxidase activity, histology and expression of muscarinic receptor isoforms using Western blot analysis. Results: Carbachol produced concentration-dependent contractions of colonic segments from control (n = 40) and TNBS-treated (n = 40) rats with no significant difference in potency. However, the maximum response to carbachol was significantly reduced in colon segments of TNBS-treated rats. The selective muscarinic receptor antagonists 4-diphenylacetoxy-N-methyl piperidine (4-DAMP, M\textsubscript{3}), pirenzepine (M\textsubscript{1}) and methoctramine (M\textsubscript{2}) antagonized carbachol-induced contraction in control (9.1 ± 0.1, 6.7 ± 0.3 and 6.0 ± 0.1, respectively) and TNBS-treated rats (9.2 ± 0.2, 6.9 ± 0.2, 6.7 ± 0.2). The –logK\textsubscript{B} values in control rats are consistent with an action of carbachol on muscarinic M\textsubscript{3} receptors. There was no significant difference in –logK\textsubscript{B} values for 4-DAMP and pirenzepine in control and TNBS-treated rats, but methoctramine was fivefold more potent in TNBS-treated rats, possibly indicating an increased contribution of muscarinic M\textsubscript{2} receptors to carbachol-induced contraction in the inflamed colon. The expression of M\textsubscript{2} receptors was also significantly increased in colon segments from TNBS-treated rats, confirming the increased role of muscarinic M\textsubscript{2} receptors in the inflamed colon. Conclusions: The data show that while only M\textsubscript{3} receptors appeared to mediate carbachol-induced contraction in control segments, expression of both M\textsubscript{2} and M\textsubscript{3} receptors was increased in the inflamed rat colon.

Introduction

Impaired carbachol-induced contraction of colon strips from 2,4,6-trinitrobenzene sulfonic acid (TNBS)-treated rats has been reported. Muscarinic receptors belong to the group of G-protein-coupled seven-transmembrane domain receptors. Muscarinic receptors are not homogeneous as five subtypes (M\textsubscript{1}–M\textsubscript{5}) have been identified [1]. All these subtypes are expressed in the gastrointestinal tract [2, 3]. Stimulation of M\textsubscript{3} receptors is associated with activation of phospholipase C with the subse-
quently hydrolysis of membrane phosphoinositol leading to the generation of inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate releases Ca^{2+} from intracellular stores, while diacylglycerol activates protein kinase C. Activation of M_{2} receptor, however, is negatively coupled to cAMP. It has been reported in several studies that the M_{3}-subtype is predominantly involved in smooth muscle contractions induced by muscarinic receptor agonists [1]. However, some studies have reported M_{2} receptor-mediated contractions in cat [4] and human [5] esophageal smooth muscle cells. In addition, a role for M_{2} receptors can be revealed under some conditions involving either inactivation of M_{3} receptors or accumulation of cAMP in the tissues since M_{3} receptors are negatively coupled to adenylyl cyclase [6, 7].

It has been reported that pathological states can alter the expression and function of muscarinic receptor subtypes. For example, an increased contribution of M_{3} receptors to carbachol-induced contractions in urinary bladder strips from rats with experimentally induced overactivity of the bladder has been reported [8]. A similar finding has been reported in inflamed canine ileum [9–11] and cat esophagus [12]. There is no information about the effect of experimental colitis on the expression and function of muscarinic receptor subtypes in the rat colon. Therefore, the main objective of this study was to examine the effect of TNBS-induced colitis on the expression and function of muscarinic receptor subtypes in the rat colon.

Materials and Methods

Male Sprague-Dawley rats (n = 80, 40 each group, 150 and 250 g) were used in this study. The animals were kept in a controlled environment and provided with standard rodent chow (Special Diet Services, England) and free access to water. All the animals were bred and maintained in the Animal Resource Center, Faculty of Medicine, Kuwait University, Kuwait.

Induction of Colitis

Colitis was induced by colonic instillation of 30 mg TNBS in 0.5 ml ethanol under light ether anesthesia. Control rats (n = 40) received an equivalent amount of the solvent. The animals were used 5 days after the injection of TNBS. Control and TNBS-treated rats (n = 40 each) were weighed before and after the induction of colitis.

Myeloperoxidase Assay

On the 5th day after TNBS administration, the rats were sacrificed and about 2.5 cm of the terminal colon was isolated and used for the assay of myeloperoxidase (MPO) activity, a marker of neutrophil infiltration into the site of inflammation. Briefly, colonic segments were finely minced and homogenized in 5 ml of 14 mM hexadecyltrimethylammonium bromide (Sigma) containing 50 mM potassium phosphate buffer (pH 6.0), with a polytron (Janke & Kunkel, Germany). The supernatants were collected by centrifugation and used to estimate MPO activity as previously described [13]. Enzyme activity was expressed as units per milligram of tissue. Enzyme unit is defined as micromoles of H_{2}O_{2} converted into product per minute per milligram of tissue at room temperature.

Contractility Experiments

Approximately 2.0-cm segments of the colon (with the mucosa intact) were cut and set up for isometric longitudinal smooth muscle contraction recording in 25.0-ml organ baths containing Krebs solution of the following composition (mM): 119 NaCl, 4.7 KCl, 2.5 CaCl_{2}, 1.2 MgSO_{4}, 1.2 KH_{2}PO_{4}, 25 NaHCO_{3} and 11 glucose (pH = 7.4). The solution was gassed continuously with 95% O_{2}, 5% CO_{2} mixture and the temperature was maintained at 37°C. Isometric responses were recorded through UFI dynamometer on a Lectromed four-channel polygraph (Multiitrace 4P). A resting tension of 1 g was applied and the tissues were allowed to stabilize for 30 min during which the bath fluid was changed at least once. After the period of equilibration, 80 mM KCl was added to the bath to test for tissue viability.

Characterization of Muscarinic Receptor Subtypes Mediating Carbachol-Induced Contraction

In order to investigate the role of muscarinic receptor subtypes in carbachol-induced contractions, the effects of selective muscarinic receptor subtype antagonists on carbachol-induced contractions were examined. In this series of experiments, increasing concentrations of carbachol were added cumulatively to generate a concentration-response curve in the absence and also in the presence of selective muscarinic receptor subtype antagonists; only one concentration of each antagonist was tested on each preparation. Briefly, after establishing the control concentration-response curve for carbachol (added cumulatively) in tissues obtained from control and TNBS-treated rats, antagonists were added to the bath and allowed to equilibrate with the tissues for 30 min before reestablishing agonist concentration-response curve. The EC_{50} values (concentration of the agonist required to give 50% of the maximum response) were used to calculate concentration ratios (CRs). Antagonist potency was expressed as the –logK_{B} where K_{B} was calculated using the equation: K_{B} = (antagonist concentration)/CR – 1.

Expression of Muscarinic Receptor Subtypes in the Colon

Colonic tissues from control and treated animals were removed and cleaned carefully with ice-cold phosphate-buffered saline. Tissue samples were weighed and used immediately or frozen in liquid nitrogen and stored at –80°C until use. Tissue samples were minced with scissors and homogenized with a polytron (Brinkman Instruments Co., Westbury, N.Y., USA) in 10 ml of ice-cold 3-(N-morpholino)propanesulfonic acid (MOPS)-sucrose buffer, pH 7.4, containing (in mmol/l) 20 MOPS, 300 sucrose and 20 EDTA. The lysates were centrifuged at 1,620 g (Beckman) for 10 min and the supernatants centrifuged at 10,000 g for 10 min (Beckman, JA-20). The supernatants were collected and centrifuged at 188,000 g for 45 min (Beckman Instruments, Palo Alto, Calif., USA). The microsome pellets so obtained were ho-
mogenized using ice-cold MOPS-sucrose buffer with a tissue homogenizer (Eberbach Corp., Mich., USA) and designated as crude microsomes.

**Western Blot Analysis**

To measure the expression of muscarinic receptor subtypes in the colon, aliquots of crude microsomes were separated on an 8% polyacrylamide gel and were then transferred electrophoretically to a nitrocellulose membrane (BioRad) overnight. After blocking with 5% non-fat milk solution in phosphate-buffered saline, the nitrocellulose membranes were incubated with monoclonal antibodies selective for the different muscarinic receptor subtypes (Santa Cruz Biotechnology, USA) for 3 h. Specific protein bands were developed using suitable secondary antibody conjugated with horseradish peroxidase (Sigma). The nitrocellulose membranes were washed thoroughly and specific bands were developed using a commercial enhanced chemiluminescence reagent kit (Amersham). Band density was estimated using a densitometer (SynGene, Chemi Genius Bio Imaging System). The expression of muscarinic receptor subtypes in colitic tissues was expressed as a percentage of the level in noncolitic controls.

**Drug Solutions**

The following drugs were used in this study: carbachol hydrochloride, pirenzepine, methoctramine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) obtained from Sigma Chemicals (USA). All the compounds were dissolved in distilled water.

**Statistical Analysis**

Data were analyzed using Graphpad Prism Software. Data were expressed as mean ± SE of n experiments where n is the number of animals used. Differences between mean values were tested for significance using paired or unpaired Student’s t test (as appropriate). The difference was considered significant when p < 0.05.

**Results**

**MPO Activity**

As shown in figure 1, MPO activity was significantly higher (p < 0.05) in colonic segments from TNBS-treated rats (23.45 ± 7.14 units/min/mg tissue, n = 5) compared to segments from control rats (0.46 ± 0.09 units/min/mg tissue, n = 5), confirming inflammation in the TNBS-treated rats used in this study.

**Contractile Response to Carbachol in Rat Colon**

Colon strips developed spontaneous rhythmic contractions within 30 min after they were set up. These rhythmic contractions were maintained throughout the whole experiment (4 h). However, the amplitude of spontaneous rhythmic contractions was greater in colon segments from control rats when compared with preparations from TNBS-treated rats. In addition, the basal tone on the tissues was also maintained throughout the duration of the experiments.

Carbachol (0.1–100 μM) produced concentration-dependent contractions of colon segments from control and TNBS-treated rats. The concentration-response curves to carbachol in colon segments from control and TNBS-treated rats are shown in figure 2. In these curves, response to all concentrations of carbachol in any particular preparation was expressed as a percentage of the maximum response produced by carbachol in that preparation. The pD₂ values were 5.9 ± 0.1 (n = 4) and 6.0 ± 0.3 (n = 3) in colon segments from control and TNBS-
treated rats, respectively. These values were not significantly (p > 0.05) different from each other. The maximum response to carbachol was significantly reduced in colon segments from TNBS-treated (4.4 ± 0.5 mg/mg tissue weight compared with 17.8 ± 1.6 mg/mg tissue weight in control rats) rats (fig. 2).

**Effect of Muscarinic Receptor Antagonists on Carbachol-Induced Contraction**

Specifically, the effects of pirenzepine (M1), methoctramine (M2) and 4-DAMP (M3) on carbachol-induced contractions were examined. As shown in figure 3, 4-DAMP (30 nM) produced a parallel rightward shift of carbachol concentration-response curve in colon segments from control and TNBS-treated rats. No suppression of the maximum response to carbachol was observed in any of the groups. The –logK_B values were 9.1 ± 0.1 (n = 6) and 9.2 ± 0.2 (n = 6) in colon segments from control and TNBS-treated rats, respectively. These values were not significantly different (p > 0.05) from each other.

Pirenzepine (3 µM) also produced a parallel rightward shift of the carbachol concentration-response curve in colon segments from control and TNBS-treated rats (fig. 4) with no suppression of the maximum response to carbachol. The –logK_B values were 6.7 ± 0.3 (n = 4) and 6.9 ± 0.2 (n = 6) in colon segments from control and TNBS-treated rats, respectively. These values were not significantly different (p > 0.05) from each other.

Methoctramine (3 µM) also produced a parallel rightward shift of the carbachol concentration-response curve in colon segments from control and TNBS-treated rats (fig. 5) with no suppression of the maximum response to carbachol. The –logK_B values were 6.0 ± 0.1 (n = 4) and 6.7 ± 0.2 (n = 6) in colon segments from control and TNBS-treated rats, respectively. These values were significantly different (p < 0.05) from each other. Methoctramine was approximately fivefold more potent against carbachol-induced contraction of colon segments from TNBS-treated rats.

**Expression of Muscarinic Receptor Subtypes in the Colon**

As shown in figures 6–8, all muscarinic receptor subtypes (M1, M2 and M3) were expressed in colon segments from control and TNBS-treated rats. The expression of M2 and M3 receptor subtypes was significantly increased in colon segments from TNBS-treated rats, while the expression of M1 receptors was decreased.

**Discussion**

A significant increase in MPO activity in colon segments from rats treated with TNBS confirmed colonic inflammation in these rats and is in agreement with previous studies [13–16]. Impaired agonist-induced contraction of colon strips is an established characteristic of coli-
Previous studies have shown that contractility of the colon segments to carbachol was depressed in rats treated with TNBS [14, 17–22]. This has been confirmed in this study. The results obtained in this study showed that carbachol induced reproducible and concentration-dependent contraction of colon segments from control and TNBS-treated rats. There was no significant difference in pD₂ values for carbachol between the two groups. This is in agreement with previous reports [14]. However, there was a significant reduction in the maximum response to carbachol in colon segments from TNBS-treated rats, confirming previous observations in this tissue [14, 17–22].

Carbachol-induced contractions of the rat colon are mediated via direct activation of muscarinic receptors since these contractions are not affected by tetrodotoxin.
but are abolished by atropine, a nonselective muscarinic receptor antagonist. Five subtypes of muscarinic receptors, M₁, M₂, M₃, M₄, and M₅, have been identified. These subtypes are expressed in the gastrointestinal tract [2, 3]. M₃ muscarinic receptors mediate the contractile response to carbachol and related agonists, while M₂-receptor-mediated contractions have been demonstrated in the guinea pig colon under conditions in which levels of cAMP have been elevated or M₃ receptors have been inactivated [6, 7]. The role of these muscarinic receptor subtypes in carbachol-induced contraction in control and TNBS-treated rats was investigated using selective subtype antagonists. The focus of this study was on M₁, M₂, and M₃ subtypes since previous studies have reported very low (or no) levels of expression of M₄ and M₅ muscarinic receptor subtypes in gastrointestinal tract smooth muscles. The results showed that 4-DAMP produced a parallel rightward shift of the concentration-response curve to carbachol in colon segments from control rats with no reduction in the maximum response, the pKₘ values, 6.7 ± 0.3 and 6.0 ± 0.1 for pirenzepine and methoctramine, respectively, were significantly lower than expected for a response mediated via M₁ and M₂ receptor subtypes, but were typical of values for an action of these antagonists on M₃ receptors [1, 8, 10, 23–25], indicating that M₁ and M₂ receptors are not involved in carbachol-induced contraction of colon segments from control rats and therefore confirming that these contractions were mediated via M₃ receptors. In colon segments from TNBS-treated rats, 4-DAMP also produced a parallel rightward shift of the carbachol concentration-response curve without reducing the maximum response to carbachol. The pKₘ value was calculated to be 4-DAMP (9.2 ± 0.1) and this was not significantly different from the corresponding value in colon segments from control rats. This would suggest that TNBS-induced colitis did not produce a change in M₃ receptor affinity/function of the colon segments. This result contrasts with that of Wells and Blennerhassett [26], who reported diminished potency of 4-DAMP in reducing acetylcholine-induced shortening of isolated smooth muscle cells from rats treated with TNBS. The reason for the difference in the potency of 4-DAMP is not known. It could, however, be due to differences in the type of preparation used – whole longitudinal colon segment (this study) versus dispersed circular smooth muscle.
smooth muscle cells [26]. This result also contrasts with that of Giglio et al. [27], who reported diminished potency of 4-DAMP in inhibiting carbachol-induced contraction of urinary bladder smooth muscle from rats with cyclophosphamide-induced cystitis. The result is, however, similar to that of Shi and Sarna [10] in ileal segments from dogs with acetic acid-induced inflammation of the ileum. Even though pirenzepine also produced a parallel rightward shift of the carbachol concentration-response curve without reducing the maximum response to carbachol, the pK_B value (6.9 ± 0.1) was within the range expected for an action on M_3 receptors, and was not significantly different from the corresponding value in colon segments from control rats. This would suggest that M_1 receptors are not involved in carbachol-induced contraction of the rat colon with or without TNBS-induced colitis. Like 4-DAMP and pirenzepine, methoctramine also produced a parallel rightward shift of the carbachol concentration-response curve in colon segments from TNBS-treated rats without reducing the maximum response to carbachol. In contrast to 4-DAMP and pirenzepine, methoctramine was about fivefold more potent (pK_B value of 6.7 ± 0.1 vs. 6.0 ± 0.1 in controls) against carbachol-induced contraction of colon segments from TNBS-treated rats compared with the controls. An increased potency of methoctramine, interpreted as an increased role for muscarinic M_2 receptors, has been reported by Wells and Blennerhassett [26] in dispersed circular smooth muscle cells of the rat colon and by Jadcherla [28] in canine colonic smooth muscle cells. Shi and Sarna [10] also made a similar observation in ileal segments from dogs with acetic acid-induced inflammation of the ileum. An increased effectiveness of methoctramine has also been reported by Sohn et al. [12] in esophageal smooth muscle of the cat. However, the pK_B value (6.7 ± 0.1) obtained in our study, though higher than the corresponding value in colon segments from control rats, was about midway between the affinities of methoctramine for M_2 and M_3 receptors, suggesting an increased contribution of muscarinic M_2 receptors in carbachol-induced contraction and not a switch from M_3 receptors to M_2 receptors mediating the contractions. An increase in the pK_B value for methoctramine coupled with a decrease in the pK_B value for 4-DAMP would suggest a switch from M_3 receptors to M_2 receptors in TNBS-treated rats.

In an attempt to relate the increase in potency of methoctramine in colon segments from TNBS-treated rats with any possible change in the density of muscarinic M_2 receptor subtype, the expression of M_2 receptor protein was examined. The expression of M_1 and M_3 receptors was also studied for comparison. The results showed that all three receptor subtypes were expressed in colon segments from control and TNBS-treated rats. However, the expression of muscarinic M_1 receptors was significantly reduced in colon segments from rats treated with TNBS, whereas the expression of muscarinic M_2 and M_3 receptors was increased in the same animals. So far, only one study reported in the literature has examined the effect of inflammation on the expression of muscarinic receptor subtypes in the gastrointestinal tract. In that study by Shi and Sarna [11], the expression of neither M_2 nor M_3 receptors was altered by acetic acid-induced inflammation despite the increased potency of methoctramine, a selective M_2 receptor antagonist. However, an increased expression of M_2 receptors has been reported in other pathological states such as hypertrophic denervated bladder [29]. Thus, our observation of an increased expression of M_2 receptors together with the increased potency of methoctramine would suggest an increased role for muscarinic M_2 receptors in carbachol-induced contractions of the inflamed rat colon. We cannot explain the increase in the expression of M_1 receptors observed in this study, but we could speculate that this might have buffered the increased potency of methoctramine and this would explain why the potency of methoctramine, though increased, was not up to what would have been expected for an action on M_2 receptors.

**Conclusion**

While only M_3 receptors appeared to mediate carbachol-induced contraction in control colon segments, both M_2 and M_3 receptors were involved in colon segments from TNBS-treated rats. The fact that carbachol-induced contraction in colon segments from TNBS-treated rats was attenuated despite the increase in expression of M_3 and M_1 receptors would suggest that the attenuation could be due to changes in postreceptor mechanisms.

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