Distribution of muscarinic receptor subtypes and interstitial cells of Cajal in the gastrointestinal tract of healthy dairy cows

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Objective—To describe the distribution of muscarinic receptor subtypes M₁ to M₅ and interstitial cells of Cajal (ICCs) in the gastrointestinal tract of healthy dairy cows.

Sample Population—Full-thickness samples were collected from the fundus, corpus, and pyloric part of the abomasum and from the duodenum, ilium, cecum, proximal loop of the ascending colon, and both external loops of the spiral colon of 5 healthy dairy cows after slaughter.

Procedures—Samples were fixed in paraformaldehyde and embedded in paraffin. Muscarinic receptor subtypes and ICCs were identified by immunohistochemical analysis.

Results—Staining for M₁ receptors was found in the submucosal plexus and myenteric plexus. Antibodies against M₂ receptors stained nuclei of smooth muscle cells only. Evidence of M₃ receptors was found in the lamina propria, in intramuscular neuronal terminals, on intermuscular nerve fibers, and on myocytes of microvessels. There was no staining for M₄ receptors. Staining for M₅ receptors was evident in the myocytes of microvessels and in smooth muscle cells. The ICCs were detected in the myenteric plexus and within smooth muscle layers. Distribution among locations of the bovine gastrointestinal tract did not differ for muscarinic receptor subtypes or ICCs.

Conclusions and Clinical Relevance—The broad distribution of M₁ to M₅ subtypes and ICCs in the bovine gastrointestinal tract indicated that these components are likely to play an important role in the regulation of gastrointestinal motility disorders in healthy dairy cows. Muscarinic receptors and ICCs may be implicated in the pathogenesis of motility disorders, such as abomasal displacement and cecal dilatation-dislocation. (Am J Vet Res 2006;67:1992–1997)

The autonomic nervous system plays an essential role in maintenance of the homeostasis of organisms. One of the major neurotransmitters in this regulatory system is acetylcholine, which acts as a ligand for 2 distinct types of receptors (ie, ionotropic nicotinic receptors and metabotropic muscarinic receptors). In the gastrointestinal tract, muscarinic receptors are involved in the regulation of motility as well as mucosal secretion. Five subtypes of the G-protein–coupled muscarinic receptors, namely M₁ through M₅, have been identified, and studies have investigated their specific modes of action.

Muscarinic receptors can be found in a wide range of organs, such as the brain (M₁ through M₅), heart (M₂), endocrine glands (M₃ and M₅), and salivary glands (M₁ and M₂). Furthermore, these receptors have been identified in the smooth muscle layers of the bladder, airways (M₂ and M₃), and intestines (M₁ through M₅) of humans, dogs, and laboratory animals. The wide distribution of various muscarinic receptor subtypes in the gastrointestinal tract is indicative of their importance for the maintenance of homeostasis and their potential involvement in the pathologic processes of diseases.

Furthermore, ICCs are important in the regulation of enteral motility. They are located in the intestinal wall in the myenteric plexus and smooth muscle layers in humans and domestic animals. The ICCs play a role in the modulation of slow-wave activity in the gastrointestinal tract and act as mediators between enteric nerves and smooth muscle cells.

Left-sided displacement of the abomasum and CDD are 2 diseases of major economic importance in dairy cows. The pathogenesis of LDA and CDD is poorly understood, and therapeutic options are often limited to surgical interventions. However, reports on the motility-enhancing effects of the parasympathomimetic drug bethanechol on the abomasum, small intestines, and large intestines of cows in vitro indicate that pharmacologic treatment may also be of value. Muscarinic receptors have been implicated in the pathogenesis of intestinal motility disorders in other species. Thus, they may also play a role in the pathogenesis of LDA, CDD, or both in cattle.

Studies to investigate the number and distribution of muscarinic receptors and ICCs in the gastrointestinal tract of cattle have not been conducted. Therefore, the objective of the study reported here was to investigate...
the distribution of muscarinic receptor subtypes and ICCs by use of immunohistochemical analysis in the gastrointestinal tract of healthy dairy cows, especially in anatomic locations relevant to the diseases of LDA and CDD.

**Materials and Methods**

**Sample population**—Tissue samples were obtained from 5 dairy cows at a local slaughterhouse within minutes after the cattle were stunned during slaughter. Full-thickness samples (approx 1.5 X 3 cm) of the gastrointestinal tract were collected from the fundus, corpus, and pyloric part of the abomasum and from the duodenum, ileum, cecum, proximal loop of the ascending colon, and 2 outermost loops of the spiral colon. Tissue samples were rinsed with PBS solution and pinned onto silicone in Petri dishes. Tissues were fixed by soaking in 4% paraformaldehyde in PBS solution for 4 hours. Tissues samples were then rinsed and processed for embedding in paraffin in accordance with standard protocols. Sections (8 μm in thickness) were placed on aminopropyltriethoxysilane-coated slides.

**Immunohistochemical analysis**—Sections were deparaffinized by use of xylol and rehydrated through a graded series of ethanol solutions. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in PBS solution for 30 minutes, and nonspecific binding of antibodies was blocked by incubation with 0.25% casein in PBS solution for 30 minutes. Endogenous peroxidase activity was quenched by incubation with ethanol solutions. Endogenous peroxidase activity was quenched by incubation with ethanol solutions. Sections were deparaffinized by use of xylol solutions. Tissues were fixed by soaking in 4% paraformaldehyde in PBS solution for 4 hours. Tissues samples were then rinsed and processed for embedding in paraffin in accordance with standard protocols. Sections (8 μm in thickness) were placed on aminopropyltriethoxysilane-coated slides.

Protocols were established for each receptor subtype and the ICCs. Protocols were initially established for tissues of known reactivity, such as rat brain (M1 and M4), rat and bovine heart (M2 and M3), bovine adrenal gland (M1), and feline footpads (ICCs). Thereafter, the same antibodies were tested in rat ileum to evaluate results in the tissue of interest in a species for which ample information about muscarinic receptor subtypes is available. After the protocols were established for use with rat intestine, they were applied to the target tissues (ie, the bovine gastrointestinal tract).

Polyclonal rabbit antibodies were used for all 5 muscarinic receptor subtypes and the ICCs to maximize binding to each specific receptor subtype (Appendix). Primary antibodies were selected on the basis of results of preliminary experiments. A number of commercially available antibodies were evaluated and validated for the species and tissues of interest.

Sections were incubated overnight with the corresponding primary antibodies for the 5 muscarinic receptor subtypes and the ICCs. Sections then were washed with PBS solution, incubated with a horseradish peroxidase–labelled polymer conjugated to goat anti-rabbit antibodies for 30 minutes, washed with PBS solution, and developed by incubation in the dark for another 30 minutes with 3,3-diaminobenzidine as a chromogen. Sections were washed, mounted with an aqueous mounting agent, and then examined by use of a microscope equipped with a digital camera.

Selection of positive control tissues relied on information published references and specificity of the antibodies as stated by the manufacturer. Negative control experiments included omission of primary antibodies and use of a rabbit anti-calcitonine antibody (1:20 dilution) on all tested tissues as a substitute for the pertinent primary antibodies.

**Figure 1**—Photomicrographs obtained after immunohistochemical staining to detect various muscarinic receptor subtypes and ICCs in tissues of the gastrointestinal tract obtained from 5 healthy cows. A—Detection of M1 in the myenteric plexus in the ascending colon. The inset of panel A reveals M1 in the intramuscular nerve fiber bundles of the pyloric part of the abomasum. B—Detection of M3 in nerve fibers in intermuscular connective tissue and neuronal terminations in pyloric smooth muscle tissue. The insets of panel B reveal M3 in the myocytes of the ileum (upper right corner) and nerve cells in the lamina propria of the cecum (lower right corner), respectively. C—Detection of M5 in smooth muscle cells of the corpus of the abomasum. The inset of panel C reveals M5 in microvessels of the corpus of the abomasum. D—Detection of M5 in smooth muscle layers of the ileum. The inset of panel D reveals M5 in the vascular tunica media of the ileum. Notice the stronger staining of the ILM, compared with that for the CML. E—Detection of ICCs in the abomasal fundus. Notice the staining of ganglia in the myenteric plexus and of single nerve cells within the muscular layers. F—Results for the negative control experiment with tissues from the duodenum. For panels A to D, tissues were incubated with rabbit antibodies against corresponding muscarinic receptors. For panels E and F, rabbit anti-c–kit and anti-calcitonine immunoglobulins were used as primary antibodies, respectively. For all panels, goat anti-rabbit antibodies were used as the second antibody. All panels were developed with 3,3-diaminobenzidine chromogen solution, and panel D was counterstained with haemalum. Bar = 50 μm. ILM = longitudinal muscle layer. CML = circular muscle layer.
Results

All antibodies yielded a strong and specific signal in accordance with known locations in positive control tissues (neurons in rat brain for M1; rat and bovine cardiac myocytes for M2 and M3; nerve fibers in the medulla and capsule of the bovine adrenal gland for M4; vascular smooth muscle cells in rat brain for M5; and epidermal squamous epithelial cells, mast cells, and sweat gland cells in feline footpads for ICCs; data not shown).

Specific staining was observed in all reference tissues and for M1, M3, and M5 in tissues obtained from the gastrointestinal tract of each of the 5 cows. Distribution patterns of these receptors remained constant throughout the gastrointestinal tract (ie, in the fundus, body and pyloric part of the abomasum and the duodenum, ileum, cecum, proximal loop, and both external [centripetal and centrifugal] loops of the spiral colon) for the respective muscarinic subtypes and the ICCs. Similarly, no differences were observed among cows.

Immunohistochemical analysis revealed that M1 was found predominantly in the submucosal plexus and myenteric plexus (ie, between the longitudinal and circular layers of the tunica muscularis; Figure 1). Furthermore, the course of M1-positive nerve fibers with distinct fiber bundles was clearly visible in the intermuscular connective tissue and within the smooth muscle layers.

Staining for M2 was consistently observed on nuclei of smooth muscle cells (data not shown). Labelling intensity was similar for the circular and longitudinal muscle layers.

Staining for M3 with the corresponding antibody was highly sensitive. This receptor subtype appeared to be located within the muscle layers (probably on neuronal terminals and nerve fibers) and in nerve cells in the lamina propria (Figure 1). Staining for M3 was also evident in myocytes of microvessels in the abomasal and intestinal wall throughout the gastrointestinal tract.

Staining for M4 was faint and inconsistent in all abomasal and intestinal segments (data not shown). Staining for M4 was prominent on smooth muscle cells (Figure 1). Staining for M4 was stronger in the longitudinal smooth muscle layer than in the circular smooth muscle layer, and staining was most evident in myocytes of microvessels in the abomasal and intestinal wall throughout the gastrointestinal tract.

Cells in the ganglia of the myenteric plexus yielded positive results when tested by use of antibodies against c-kit, which indicated evidence of ICCs. In addition, single cells within the smooth muscle layers also had positive results for c-kit antibodies (Figure 1).

Staining was not evident when the anti-calcitonine primary antibody was used instead of the primary antibodies for the various muscarinic receptor subtypes and the ICCs (Figure 1).

Discussion

Postganglionic nerve fibers of the parasympathetic nervous system, together with ICCs, play a crucial role in promoting intestinal motility.17 Nerve cells in the plexus of the intestinal wall influence several functions of the gastrointestinal tract, including muscular contraction or relaxation and secretion or absorption.20 Accordingly, muscarinic receptors may also be involved in the pathogenesis of motility disorders, such as LDA and CDD, in dairy cows. However, the distribution of muscarinic receptor subtypes and ICCs in the bovine digestive tract has not been investigated. Therefore, the study reported here was designed to use immunohistochemical analysis to establish the distribution of muscarinic receptor subtypes (M1 to M5) and ICCs in various regions of the gastrointestinal tract of healthy cattle. In conjunction with current knowledge on the effects of these receptors, the data reported here provide a basis to elaborate functional hypotheses and to design functional and pharmacologic investigations that will contribute to a better understanding of the role of muscarinic receptor subtypes and ICCs in the gastrointestinal tract of cattle.

To our knowledge, specific antibodies for bovine muscarinic receptors and ICCs have not been developed. However, these proteins are widely conserved among species,21 and we selected antibodies from a number of commercially available products. Antibodies were thoroughly validated on organs from species with known reactivity as well as on corresponding bovine tissues. Only antibodies yielding accepted labelling patterns in bovine control organs were used to investigate muscarinic receptors and ICCs in the gastrointestinal tract of cattle.

Staining for M1 was mainly found in the submucosal plexus and myenteric plexus, and staining of nerve bundles was also observed within smooth muscle layers. Cells in the nervous plexus of the intestinal wall influence several functions of the gastrointestinal tract, including muscular contraction and relaxation as well as secretion and absorption.22 The fact that M1 was found in the nervous plexus of the intestinal wall as well as within the musculature of the bovine abomasum and intestines indicates that it may play a pivotal role in the modulation of gastrointestinal activity in this species.

Detection of M2 in the plasmalemma of bovine intestinal smooth muscle cells was anticipated because M2 and M3 have been reported in a ratio of 4:1 or higher in a number of species. Instead, labelling of bovine gastrointestinal tissue with the anti-M2 antibody was restricted to the cell nuclei of myocytes in the muscle layers. Nuclear staining was also detected in positive control tissues. However, in rat and bovine cardiac tissues, additional labelling was consistently observed at the periphery of myocytes. This reaction was considered to be specific because it was abolished when anti-calcitonine antibody was substituted for the appropriate primary antibody. Thus, species differences in epitope conformation are not the reason for the lack of staining in the periphery of intestinal smooth muscle cells.

The failure to detect M2 at the periphery of bovine intestinal smooth muscle cells is troubling. It may reflect a true lack of or low density of this receptor subtype in the bovine gastrointestinal tract, or it may be indicative of further diversity of the receptor configuration in various organs.
In most species, density of M₃ is low when compared with the density of M₂. However, M₃ appears to play the most important role in smooth muscle contraction.²⁵,²⁶ In the study reported here, intensive staining was observed for M₁ in intramuscular nerve fibers as well as in neurons of the lamina propria. Such locations are compatible with an effect of these receptors on intestinal motility. A further effect on vasculature is suggested by the fact that this subtype was also found in the myocytes of vessel walls.

Despite reliable evidence of M₄ in bovine adrenal glands, this receptor was not detected in the bovine gastrointestinal tract. The M₅ has been detected in cultures of muscle cells obtained from the esophagus of humans,³⁹ smooth muscle of the stomach of guinea pigs,³⁸ and ileum of rabbits.³⁹ However, M₄ is also lacking in the colon of humans.³⁹

The distribution and function of M₅ in the smooth muscle of the gastrointestinal tract has received little attention, although it has been identified in smooth muscle in the esophagus of humans and stomach of guinea pigs.²⁶ In the study reported here, M₅ was identified on the surface of smooth muscle cells in both muscle layers of the gastrointestinal tract, with stronger staining in the longitudinal muscle layer than in the circular muscle layer. This finding suggests a role of M₅ in the regulation of muscle contraction or relaxation in the bovine gastrointestinal tract. Staining for M₅ was also observed in myocytes in the walls of microvessels.

Immunohistochemical analysis conducted by using the anti–c-kit antibody has been used in a wide range of species to study the distribution of ICCs in the digestive tract. Evidence of ICCs between the muscle layers, similar to the results observed in the study reported here, has also been reported in mice,³ guinea pigs,³⁸ and horses.²⁰ This suggests a potential colocalization of ICCs with M₅ in the myenteric plexus. Furthermore, staining of intramuscular nerve fibers was observed for ICCs and M₅, which may indicate expression of this muscarinic receptor subtype on ICCs. Thus, potential functional interactions between muscarinic receptors and ICCs warrant further investigation.

Both diseases of interest, LDA and CDD, are believed to be triggered by a primary motility disturbance in the corresponding segments of the digestive tract.³⁵,⁴¹ Because muscarinic receptors are involved in the regulation of gastrointestinal tract motility, they may also play a role in the pathogenesis of motility disorders.¹⁵ Indeed, several muscarinic receptor agonists have been considered for use in the treatment of humans and horses¹⁵ with motility disorders, and the use of the direct acting M₁- and M₃-agonist bethanechol has been advocated for medical treatment of cows with CDD.³⁵ Because prokinetic effects in the intestines are believed to be caused primarily by activation of M₃ rather than M₂,¹³,¹⁴ the failure to detect M₂ in the bovine gastrointestinal tract does not necessarily preclude a potential positive therapeutic effect of bethanechol in cattle with motility disorders.

Although M₁ and M₃ are usually considered to play the most prominent role in gastrointestinal tract motility,¹³ the wide distribution of M₁ in the bovine gastrointestinal tract suggests that this receptor subtype may also be of great importance in cattle. This is in agreement with the observations that the effect of the indirect parasympathomimetic drug neostigmine in humans is mediated through stimulation of M₁ in the myenteric plexus³⁴ and that this receptor subtype conveys inhibitory effects on excitability of the proximal portion of the intestines in dogs.³⁴

The distribution and function of M₃ in the gastrointestinal tract have received little attention,³³ however, in the study reported here, M₃ was identified at the surface of smooth muscle cells in both muscle layers of the gastrointestinal tract. This makes M₃ another candidate for a role in the regulation of muscle contraction or relaxation in this species. Furthermore, evidence suggests that the blood supply is modulated through cholinergic neurons.⁹,¹⁰ In our study, M₃ and M₅ were detected in the smooth muscle layer of microvessels throughout the abomasal and intestinal walls of the bovine gastrointestinal tract. Analysis of these results indicates that these receptor subtypes are likely to be involved in the regulation of vascular tone in the wall of gastrointestinal organs.

Muscarinic receptor subtypes M₁, M₃, and M₅ were identified by use of immunohistochemical analysis on neurons and smooth muscle cells as well as vessel walls (for M₁ and M₃) of the gastrointestinal tract of healthy dairy cows. Staining for ICCs also was evident in all intestinal segments included in the study, which extended from the abomasum to the colon. On the basis of analysis of these results, additional studies are warranted to determine the relationship between muscarinic receptors and ICCs. A possible implication of 1 or several muscarinic receptor subtypes in the pathogenesis of LDA and CDD should be investigated in studies conducted on the expression of these receptors in the digestive tract of healthy and affected cattle. Future studies should also address functional aspects, such as the subtype specificity of prokinetic drugs (eg, bethanechol), to document the precise mechanism of action of these compounds, assuming they are to be used for treatment and control of motility disorders. Effective medical treatment of motility disorders, such as LDA and CDD, should reduce the need for surgical treatment of these diseases or at least improve postoperative management, thus shortening recovery time and reducing relapse rates.

a. PBS tablets, Calbiochem, Bern, Switzerland.
b. Protein block, serum-free, DakoCytomation GmbH, Wien, Austria.
c. Coverplate, Thermo Shandon, Zug, Switzerland.
d. Anti-muscarinic acetylcholine receptor M₁ (M9808), Sigma-Aldrich Inc, Buchs, Switzerland.
e. Anti-muscarinic acetylcholine receptor M₂ (M9538), Sigma-Aldrich Inc, Buchs, Switzerland.
f. Muscarinic acetylcholine receptor M₅ antibody (NLS5259), Novus Biologicals, Montluçon, France.
g. Muscarinic acetylcholine receptor M₃ antibody (NLS219), Novus Biologicals, Montluçon, France.
h. Muscarinic acetylcholine receptor M₄ antibody (NLS1338), Novus Biologicals, Montluçon, France.
i. Anti–c-kit (961-976) rabbit pAb (PC34), Oncogene Research Products, San Diego, Calif.
j. Envisons System anti-rabbit, DakoCytomation GmbH, Wien, Austria.

k. Liquid DAB substrate chromogen system, DakoCytomation GmbH, Wien, Austria.

l. Aquatex, Merck KGaA, Darmstadt, Germany.

m. Zeiss Axioskop 2 with AxiosCam HR, Carl Zeiss AG, Feldbach, Switzerland.

n. Rabbit anti-calcitonin serum (1720-7904), Anawa, Zurich, Switzerland.

References


**Appendix**

Primary rabbit antibodies, their characteristics, and the dilutions at which they were used to test tissues obtained from the gastrointestinal tract of 5 cows for various muscarinic receptor subtypes and ICCs.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Antigen</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-M1</td>
<td>Polyclonal</td>
<td>Third intracellular loop of the human M1 AChR</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-M2</td>
<td>Polyclonal</td>
<td>Third intracellular loop of the human M2 AChR</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-M3</td>
<td>Polyclonal</td>
<td>Third cytoplasmic loop of the human M3 AChR</td>
<td>1:400</td>
</tr>
<tr>
<td>Anti-M4</td>
<td>Polyclonal</td>
<td>Third cytoplasmic loop of the human M4 AChR</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-M5</td>
<td>Polyclonal</td>
<td>Third cytoplasmic loop of the human M5 AChR</td>
<td>1:400</td>
</tr>
<tr>
<td>Anti–c-kit</td>
<td>Polyclonal</td>
<td>Amino acids 961 to 976 of the C-terminus</td>
<td>1:100</td>
</tr>
</tbody>
</table>

AChR = Acetylcholine receptor.