In vitro effects of bethanechol on specimens of intestinal smooth muscle obtained from the duodenum and jejunum of healthy dairy cows

Julia B. R. Pfeiffer, Dr med vet; Meike Mevissen, Dr med vet, Dr habil; Adrian Steiner, Dr med vet, MS, Dr habil; Christopher J. Portier, PhD; Mireille Meylan, Dr med vet, PhD, Dr habil

Objective—To describe the in vitro effects of bethanechol on contractility of smooth muscle preparations from the small intestines of healthy cows and define the muscarinic receptor subtypes involved in mediating contraction.

Sample Population—Tissue samples from the duodenum and jejunum collected immediately after slaughter of 40 healthy cows.

Procedures—Cumulative concentration-response curves were determined for the muscarinic receptor agonist bethanechol with or without prior incubation with subtype-specific receptor antagonists in an organ bath. Effects of bethanechol and antagonists and the influence of intestinal location on basal tone, maximal amplitude (A_max), and area under the curve (AUC) were evaluated.

Results—Bethanechol induced a significant, concentration-dependent increase in all preparations and variables. The effect of bethanechol was more pronounced in jejunal than in duodenal samples and in circular than in longitudinal preparations. Significant inhibition of the effects of bethanechol was observed after prior incubation with muscarinic receptor subtype M_3 antagonists (more commonly for basal tone than for A_max and AUC). The M_3 receptor antagonists partly inhibited the response to bethanechol, especially for basal tone. The M_2 receptor antagonists were generally more potent than the M_3 receptor antagonists. In a protection experiment, an M_2 receptor antagonist was less potent than when used in combination with an M_3 receptor antagonist. Receptor antagonists for M_1 and M_4 did not affect contractility variables.

Conclusions and Clinical Relevance—Bethanechol acting on muscarinic receptor subtypes M_1 and M_3 may be of clinical use as a prokinetic drug for motility disorders of the duodenum and jejunum in dairy cows. (Am J Vet Res 2007;68:313–322)

The important role of the parasympathetic nervous system in physiologic processes of GI tract motility and in the pathophysiology of motility disorders has been described for various species. In the cholinergic system, acetylcholine activates G-protein-coupled muscarinic receptors to cause smooth muscle contraction in several organs. Five mAChR subtypes (M_1 to M_5, respectively) have been cloned and defined pharmacologically.

The M_1 and M_3 mAChR subtypes are the predominant receptors in the GI tract of several species, with a ratio for M_1/M_3 of approximately 4:1. However, the M_2 receptor subtype is considered to be of predominant importance in eliciting muscle contractions, and the exact role of the more abundant M_3 subtype remains unclear. A study in M_1 receptor knockout mice has emphasized the role of M_1 mAChRs for smooth muscle contraction because ileal contractile response to carbachol in vitro was reduced by 75% as compared to control animals. In comparison, ileal responses to carbachol were only slightly depressed in smooth muscle tissues from M_1 knock-out mice. In contrast to these in vitro findings, M_1 knock-out mice, as well as M_2- and M_3 knock-out mice, were free of apparent GI tract disorders, which suggests redundant regulation mechanisms through other mAChR subtypes or other mediators.

In addition to M_1 and M_3, other mAChR subtypes are expressed in GI tissues. The M_2 to M_5 mAChRs...
have been detected in the digestive tract of several species, such as humans, pigs, dogs, mice, rats, guinea pigs, and rabbits.\textsuperscript{10,15-22} Immunohistochemical analysis has been used to verify \( M_1, M_3, \) and \( M_4 \) mAChRs in the bovine GI tract.\textsuperscript{23} In that study, results for \( M_3 \) mAChRs were inconclusive, and \( M_4 \) mAChRs could not be detected.

Loss of intestinal tone and motility resulting in failure of orocaudal movement of GI contents and accumulation of fluid, gas, and ingesta that causes intestinal distention and abdominal pain is a life-threatening condition.\textsuperscript{24} In contrast to findings in horses, spontaneous paralytic (adynamic) ileus is more common in cattle than is postoperative paralytic ileus.\textsuperscript{5,24} Although the factors involved in the pathogenesis of postoperative ileus include inflammatory, metabolic, hormonal, neurogenic, and vascular mechanisms,\textsuperscript{25} spontaneous paralytic ileus in cattle is mainly attributable to metabolic disturbances and electrolyte imbalances, such as hypokalemia or hypocalcemia,\textsuperscript{6,26} but sympathetic hyperactivity may also be involved.\textsuperscript{24}

The duodenum also plays a pivotal role in abomasal emptying in ruminants; hence, motility disorders of the duodenum may result in incomplete abomasal emptying. This is believed to be associated with abomasal displacement, a common problem in dairy cattle.\textsuperscript{27,28} Therefore, prokinetic drugs that act on the small intestines and abomasum in cattle may be useful for clinical management of hypomotility or atony of the intestines and abomasal displacement.

Bethanechol, a methyl derivative of carbachol, induces contraction of smooth muscle cells by direct stimulation of mAChRs.\textsuperscript{2,29,30} It has been reported that there are no muscarinic receptor agonists with an extremely high selectivity for any particular mAChR subtype;\textsuperscript{6} however, bethanechol was found to act primarily via \( M_1 \) and \( M_3 \) mAChR subtypes.\textsuperscript{31}

Several researchers have indicated that bethanechol may be a promising substance for use in the treatment of animals with GI motility disorders. Bethanechol stimulates gastric emptying and intestinal propulsion in rats.\textsuperscript{32} In healthy horses, bethanechol stimulates gastric emptying and myoelectric activity of the ileum, cecum, and right ventral colon.\textsuperscript{33} In vitro, bethanechol induces a significant concentration-dependent increase in contractility traits in preparations of smooth muscle obtained from the esophageal groove of calves\textsuperscript{35} as well as the abomasal antrum\textsuperscript{35} and proximal portion of the colon of healthy cows.\textsuperscript{36} In contrast, no contractile effect of bethanechol was observed in preparations of smooth muscle obtained from the proximal portion of the duodenum.\textsuperscript{35} In vivo, bethanechol increases the myoelectric activity of the ileoceccolic area of healthy cows\textsuperscript{37} and the abomasum and duodenum of healthy yearling cattle.\textsuperscript{27} Bethanechol (0.07 mg/kg, SC, q 8 h for 2 days) also reportedly can be used successfully for conservative treatment of cows with spontaneous ceical dilatation.\textsuperscript{38} To our knowledge, in vitro effects of bethanechol on contractility of smooth muscle obtained from the distal portion of the duodenum and jejunum of cows and the various mAChR subtypes involved in mediating contraction have not been investigated.

The purpose of the study reported here was to determine whether bethanechol has an in vitro effect on contractility variables of longitudinal and circular smooth muscle obtained from the duodenum and jejunum of healthy cows and whether mAChR antagonists vary in their effects on the response induced by bethanechol. These results may also indicate the mAChR subtypes that are functionally involved in cholinergic contraction of smooth muscle in the distal portion of the duodenum and jejunum of cattle.

Materials and Methods

Tissue samples—Tissue samples were obtained at an abattoir from dairy cows that did not have a history of GI tract disease. Samples were obtained from 40 dairy cows and randomly allocated to 4 experimental groups. Breed and age distribution of the cows was similar for all experimental groups.

Specimens were collected within 20 minutes after cattle were stunned. Samples from the duodenum were obtained at a point 50 cm aboral to the pylorus, and jejunal samples were obtained at a point 2 m oral to the ileocecal valve. Tissue samples (approx 8 cm) were immediately rinsed with cool (4°C) modified Krebs solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 2.5 mM MgSO\(_4\), 3.3 mM CaCl\(_2\), 25 mM NaHCO\(_3\), and 12.2 mM glucose monohydrate) that had been oxygenated with carbogen (95% oxygen and 5% carbon dioxide)\textsuperscript{39} for 1 hour before use. Tissue samples were placed into this solution during transport to the laboratory (approx 20 minutes). At the laboratory, rectangular tissue samples were pinned flat in a dissecting dish containing oxygenated modified Krebs solution and cut parallel to the circular and longitudinal muscle fibers, respectively, by use of a custom-designed scalpel with 2 parallel blades. Final size of the resulting preparations was approximately 3 × 8 mm. Finally, the intestinal mucosa was removed from the preparation.

Recording of data—Muscle preparations (DC, DL, JC, and JL) were suspended separately in organ bath chambers,\textsuperscript{4} each of which contained 50 mL of modified Krebs solution (37°C) constantly oxygenated with carbogen. Preparations were randomly allocated to the organ bath chambers. The distal end of each muscle strip was clamped to a hook and the proximal end was attached to an isometric force transducer,\textsuperscript{4} as described elsewhere.\textsuperscript{31}

The mechanical response of the muscle strips was amplified and recorded on a personal computer by use of a commercial data acquisition system.\textsuperscript{2} The sampling rate was set at 10 samples/s/channel.

Experimental protocol—Preparations of smooth muscle were allowed to equilibrate for 1 hour, which included a period of 20 minutes of adjustment without tension, followed by a period of 1 g of tension for 10 minutes and a period of 2 g of tension for another 30 minutes. The functional viability of muscle preparations was assessed at the beginning and end of each experiment by the addition of carbachol\textsuperscript{i} (1 × 10\(^{-5}\)M) to control for muscle contraction. Only specimens that responded to carbachol with an increase in contractility variables were included in the study.
Carbachol application at the beginning of each experiment was followed by 3 flushes (whereby modified Krebs solution was completely changed 3 times at 15-second intervals in each organ bath) and a 15-minute recovery period. The subsequent 3 minutes were defined as the predrug period. Subsequently, cumulative concentration-response curves for bethanechol were generated for each tissue location and orientation by increasing the concentration of bethanechol in each organ bath in logarithmic steps from 10^{-7} to 10^{-1}M at 5-minute intervals. In each experiment, these concentration-response curves were generated with bethanechol, alone or after specimens had been incubated for 20 minutes with antagonists for various mAChR subtypes, which was designed to test the ability of each receptor antagonist to inhibit or enhance contraction generated by the cumulative administration of bethanechol. Each cumulative concentration-response curve was followed by another 3 flushes with modified Krebs solution, a 15-minute recovery period, and a 5-minute predrug period for the subsequent measurement. The order of reversibly binding mAChR antagonists was assigned randomly, but irreversibly binding receptor antagonists and atropine were always tested at the end of the experimental series.

Four experiments were performed. In experiment 1, the specimens (n = 10) were incubated with 11-[{diethylamino}(methyl)-1-piperidinyl(acyetyl)-3,11-di-hydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one (AF-DX 116; 10^{-3}M), a reversible M_{3} mAChR antagonist, and with 1.1-dimethyl-4-diphenylacetoxytropinepiperidinium iodide (4-DAMP; 2 × 10^{-3}M), an irreversibly binding M_{1} mAChR antagonist. In experiment 2, the specimens (n = 10) were incubated with N,N’-bis-[6-[(2-methoxyphenyl)methyl]amino]-1,8-octane diamine tetrahydro-sila-difenidol hydrochloride, p-F-HHSiD (experiment 3, the specimens (n = 10) were incubated with 11-[{2,3-bis-[6-[(2-methoxyphenyl)methyl]amino]-1,8-octane diamine tetrahydro-dichloride (methoctramine tetrahydrochloride); 3 × 10^{-3}M), a receptor antagonist targeting M_{1} and M_{3} mAChR subtypes; cyclohexyl-(4-fluorophenyl)-[3-(N-piperidinopropyl)]silanol hydrochloride (p-fluorohexydro-sila-difenidol hydrochloride, p-F-HHSiD; 8 × 10^{-3}M), an M_{1} receptor antagonist; and atropine sulfate (10^{-3}M), a nonselective mAChR antagonist. In experiment 4, the specimens (n = 10) were incubated with 5,11-dihydro-11-[{4-methyl-1-piperazinyl(acyetyl)-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one dihydrochloride (pirezepine dihydrochloride); 10^{-3}M], an M_{1} mAChR antagonist, and with a combination of AF-DX 116 (10^{-4}M) and 4-DAMP (2 × 10^{-3}M). In experiment 4, a cumulative concentration-response curve for bethanechol was recorded for specimens (n = 10) incubated with N-ethyl-3-hydroxy-2-phenyl-N-(pyridinylmethyl) propanamide (tropicamide; 5 × 10^{-3}M), an M_{3} mAChR antagonist. Then, the muscle strips were subjected to a protection assay (termed 4-DAMP experiment). Specimens were incubated for 1 hour with 4-DAMP (2 × 10^{-3}M) and AF-DX 116 (10^{-4}M); the reversible receptor antagonist AF-DX 116 was used to protect M_{3} mAChRs during the alkylation procedure of M_{1} mAChRs induced by 4-DAMP. Tissue specimens were then flushed 3 times, and the cumulative concentration-response curves for bethanechol were recorded.

At the end of each experiment, muscle specimens were dried between 2 absorbent paper towels, and weight and length of each specimen were measured. All substances were dissolved in distilled water, except for AF-DX 116, 4-DAMP, and tropicamide, which were dissolved in dimethyl sulfoxide. All concentrations were expressed as final concentrations in the organ baths.

**Data analysis and statistical analysis**—Several contractility variables were analyzed, which included basal tone (the tension measured between contractions [ie, the minimal amplitude], which was determined for each step of the concentration-response curves), A_{max}, and AUC. Variables were calculated at 5-minute intervals for each preparation and concentration by use of commercial software. Calculated values for basal tone, A_{max}, and AUC were then adjusted by dividing them by the corresponding value of the cross-sectional area of the muscle specimens. Cross-sectional area was calculated for each preparation by assuming a tissue density of 1.056 g/cm^{2} and by use of the following equation:

\[ \text{area} = \text{mass}(\text{density} \times \text{length}) \]

Concentration-response curves were calculated by use of the Hill equation and estimation by use of a least squares method with commercial simulation software.

The underlying equation for the Hill function was as follows:

\[ \text{Response} = \frac{(V_{\text{max}} \times C^{\alpha})}{(C^{\alpha} + K^{\alpha})} \]

where C is the compound concentration, K is the EC_{50}, and \alpha describes the shape of the function (Hill coefficients with values > 1 describe curves with a flat low-dose region and high curvature, whereas values < 1 correspond to curves that increase rapidly). Significance of comparisons made on the basis of this model was determined by use of the likelihood ratio statistic, which yielded a \chi^{2} test. The SD values for variables in the model were based on the Cramer-Rao statistic. Results were expressed as V_{max} and EC_{50} of basal tone, A_{max}, and AUC, respectively.

Results of the 4 experiments were combined to investigate various treatment effects and were analyzed in 3 separate analyses.

For analysis 1, the effect of cumulative concentrations of bethanochol was tested with the Friedman test for each intestinal location and muscle orientation separately by use of commercial software. Differences in V_{max} and EC_{50} values for the bethanochol concentration-response curves without prior incubation with antagonists for all experiments (n = 40) were compared among intestinal locations and muscle orientations for basal tone, A_{max}, and AUC by use of the \chi^{2} test.

For analysis 2, V_{max} and EC_{50} values of the bethanochol concentration-response curves with or without mAChR antagonists were compared for basal tone, A_{max}, and AUC within each experiment (n = 10) and for each combination of intestinal location and muscle orientation (DC, DL, JC, and JL) separately by use of the \chi^{2} test. Effects of the antagonists were compared between AF-DX 116 and 4-DAMP (experiment 1) and methoctramine and p-F-HHSiD (experiment 2) in accordance with the same principle.

For analysis 3, V_{max} and EC_{50} values of the bethanochol concentration-response curves for incubation with AF-DX 116 (experiment 1; n = 10), 4-DAMP (ex-
The combination of AF-DX 116 and 4-DAMP (experiment 3; 10), and 4-DAMP$_{prenax}$ (experiment 4; 10) were compared for $A_{\text{max}}$ and AUC for each intestinal location and muscle orientation separately by use of the $\chi^2$ test. Results of $V_{\text{max}}$ for $A_{\text{max}}$ and AUC were expressed relative to the corresponding predrug period of the concentration-response curves of bethanechol when incubated with the specific receptor antagonist and to the corresponding values of the bethanechol concentration-response curve without prior incubation with antagonists to correct for possible differences among experiments.

For analyses 1 and 2, results of $V_{\text{max}}$ for basal tone were reported as absolute values, whereas values for $A_{\text{max}}$ and AUC were expressed as proportions relative to the corresponding predrug values. For all comparisons, significant differences were defined as values of $P < 0.05$.

**Results**

Comparisons among intestinal locations and muscle orientations for concentration-response curves of bethanechol (analysis 1)—In all preparations, bethanechol induced a significant ($P < 0.001$) concentration-dependent increase in basal tone. Values for $V_{\text{max}}$ differed significantly among locations within orientations and were significantly higher in preparations from the jejunum than from the duodenum. The value for JC (20.2 g/cm$^2$) was significantly ($P = 0.018$) higher than the value for DC (13.5 g/cm$^2$), and the value for JL (31.7 g/cm$^2$) was significantly ($P < 0.001$) higher than the value for DL (13.7 g/cm$^2$). Within the jejunum, $V_{\text{max}}$ was significantly ($P = 0.016$) higher in longitudinal specimens (JL, 31.7 g/cm$^2$), compared with the value for the circular preparations (JC, 20.2 g/cm$^2$); results for the longitudinal and circular samples did not differ significantly within the duodenum. The EC$_{50}$ values did not differ significantly among locations and orientations.

A significant ($P < 0.001$) concentration-dependent increase in AUC and V$_{\text{max}}$ was observed after bethanechol application in all preparations (Figure 1). Significant differences among locations and orientations were similar for $A_{\text{max}}$ and AUC. Within locations, $V_{\text{max}}$ was greater in circular than in longitudinal preparations. Value for $A_{\text{max}}$ in the DC (18.4 g/cm$^2$) was significantly ($P = 0.001$) higher than the value for DL (6.0 g/cm$^2$), whereas $A_{\text{max}}$ in the JC was 18.3 g/cm$^2$, which was higher but not significantly ($P = 0.090$) different from $A_{\text{max}}$ in the JL (12.5 g/cm$^2$). The AUC value for DC (13.4 g/cm$^2$) was significantly ($P < 0.001$) higher than the value for DL (3.1 g/cm$^2$). Similarly, the AUC value for JC (15.7 g/cm$^2$) was significantly ($P = 0.003$) higher than the value for JL (8.4 g/cm$^2$).

Comparison between locations within longitudinal preparations revealed that $V_{\text{max}}$ values were significantly higher in preparations from the jejunum than in preparations from the duodenum. The $A_{\text{max}}$ for the JL (12.5 g/cm$^2$) was significantly ($P = 0.014$) higher than the value for the DL (6.0 g/cm$^2$), and the AUC for the JL (8.4 g/cm$^2$) was significantly ($P < 0.001$) higher than the AUC for the DL (3.1 g/cm$^2$). No significant differences were found between locations within circular preparations. Significant differences were not found in EC$_{50}$ values among locations and orientations.

Effects of antagonists specific for various mAChR subtypes on bethanechol-induced contractile responses (analysis 2)—Prior incubation with atropine almost completely abolished the effect of bethanechol for all variables in all preparations. The $M_1$ mAChR antagonist pirenzepine and $M_4$ mAChR antagonist tropicamide had no significant effects on the bethanechol response for any variable. Bethanechol concentration-response curves determined for $A_{\text{max}}$ for circular samples from the duodenum incubated with and without pirenzepine and tropicamide were plotted (Figure 2).

For basal tone, prior incubation with the $M_4$ mAChR antagonist 4-DAMP caused a significant decrease in $V_{\text{max}}$ in all preparations and a significant increase in EC$_{50}$ values in circular and longitudinal duodenal samples, compared with results for bethanechol alone (Table 1). Incubation with the $M_1$ mAChR antagonist AF-DX 116 significantly decreased $V_{\text{max}}$ and higher EC$_{50}$ values were apparent in the duodenal samples, compared with results for bethanechol alone. The inhibiting effect of 4-DAMP ($M_4$ receptor antagonist) on $V_{\text{max}}$ was significantly more pronounced than was the inhibiting effect of AF-DX 116 ($M_1$ receptor antagonist) in all preparations, whereas no significant differences were observed in EC$_{50}$ values. The combination of AF-DX 116 and 4-DAMP caused a significant decrease in $V_{\text{max}}$ in all preparations, compared with results for bethanechol alone, whereas significant differences were not found for EC$_{50}$ values. For basal tone, 4-DAMP$_{prenax}$ significantly inhibited the response to bethanechol for $V_{\text{max}}$ (JC, $P < 0.001$; JL, $P = 0.006$) and EC$_{50}$ (DC, $P < 0.001$; JL, $P = 0.002$).

The $M_4$ mAChR antagonist p-F-HHSiD caused a significant decrease in $V_{\text{max}}$ for basal tone in all preparations, compared with results for bethanechol (Figure 3). The EC$_{50}$ values were significantly increased in circular ($P = 0.005$) but not in longitudinal ($P = 0.089$) specimens from the jejunum (Table 1). The $M_1$ mAChR antagonist methoctramine had a significant inhibitory

![Figure 1](image1.png)
Because several premax values in ECoctramine caused lower but not significantly (*P* = 0.076; AUC for DC, *P* = 0.044), compared with results for methoctramine alone. However, EC_{so} values did not differ significantly (*A*_{max} for JC, *P* = 0.006; AUC for DL, *P* = 0.072), compared with results for methoctramine alone. Similarly, there was a significant decrease for the AUC value for DC (*P* < 0.001) and JC (*P* = 0.044), compared with results for methoctramine alone. Therefore, EC_{so} values were not observed in EC_{octramine} values.

The combination of AF-DX 116 and 4-DAMP caused a significant decrease in V_{max} in circular samples from both locations for A_{max} and AUC (*A*_{max} for DC, *P* = 0.006; A_{max} for JC, *P* < 0.001; AUC for DC, *P* = 0.004; and AUC for JC, *P* < 0.001). No significant differences were found among EC_{so} values.

The 4-DAMP caused a significant decrease in V_{max} for A_{max} (JC, *P* = 0.049) but a nonsignificant decrease in V_{max} for AUC (JC, *P* = 0.061), compared with results for methoctramine alone (Table 2). The 4-DAMP significantly inhibited the response to methoctramine for both contractility variables for EC_{so} (*A*_{max} for DC, *P* = 0.009; A_{max} for JC, *P* = 0.011; and AUC for DC, *P* = 0.010). No significant differences were detected for all other comparisons.

The receptor antagonists AF-DX 116, methoctramine, and p-F-HHSSiD did not significantly influence the effect of methoctramine on A_{max} and AUC (Table 2). We did not detect a significant difference for V_{max} or EC_{so}, when effects of methoctramine were compared with effects of p-F-HHSSiD.

Comparison of the effects of AF-DX 116, 4-DAMP, and 4-DAMP_{max} (analysis 3)—Because several predrug values for basal tone were extremely low, it was not possible to transform the values relative to the respective bethanechol curves and the predrug values to conduct analysis 3. Consequently, comparisons among receptor antagonists were conducted for A_{max} and AUC only.

The EC_{so} values for A_{max} were significantly lower for 4-DAMP_{max} than for 4-DAMP alone (JC, *P* = 0.047) or after combined incubation with AF-DX 116 and 4-DAMP (DC, *P* = 0.003; JC, *P* = 0.025), whereas no significant differences in V_{max} were observed for the same comparisons. There was a nonsignificant effect for A_{max}, in 1 location each for EC_{so} values (JC, *P* = 0.093), which indicated a less pronounced inhibiting effect for 4-DAMP_{max}, than for AF-DX 116 alone. Similarly, there was a nonsignificant effect (DL, *P* = 0.059), which indicated a more pronounced inhibiting effect for AF-DX...
Table 1—Mean ± SD values for \( V_{\text{max}} \) and \( EC_{50} \) values for various mAChR antagonists on bethanechol concentration-response curves for basal tone for DL.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>mAChR agonist or antagonist</th>
<th>Primary selectivity</th>
<th>( V_{\text{max}} ) (g/cm²)</th>
<th>( EC_{50} ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bethanechol</td>
<td>NA</td>
<td>25.52 ± 28.83</td>
<td>2.62 \times 10^{-4} ± 3.34 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + AF-DX 116</td>
<td>M₂</td>
<td>22.01 ± 76.59</td>
<td>1.00 \times 10^{-4} ± 7.25 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + 4-DAMP</td>
<td>M₃</td>
<td>4.45 ± 19.71</td>
<td>1.00 \times 10^{-4} ± 1.25 \times 10^{-4}</td>
</tr>
<tr>
<td>2</td>
<td>Bethanechol</td>
<td>NA</td>
<td>12.13 ± 14.47</td>
<td>2.59 \times 10^{-4} ± 4.24 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + methoctramine</td>
<td>M₃ and M₄</td>
<td>12.62 ± 46.47</td>
<td>1.00 \times 10^{-4} ± 4.81 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + p-F-HHSiD</td>
<td>M₃</td>
<td>1.72 ± 35.32</td>
<td>3.40 \times 10^{-4} ± 3.38 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + atropine</td>
<td>Nonselective</td>
<td>0.13 ± 0.31</td>
<td>1.13 \times 10^{-4} ± 1.59 \times 10^{-4}</td>
</tr>
<tr>
<td>3</td>
<td>Bethanechol</td>
<td>NA</td>
<td>17.95 ± 20.66</td>
<td>2.54 \times 10^{-4} ± 1.24 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + tropicamide</td>
<td>M₃</td>
<td>24.52 ± 57.36</td>
<td>6.04 \times 10^{-4} ± 2.68 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + AF-DX 116</td>
<td>M₃ and M₄</td>
<td>3.02 ± 9.91</td>
<td>5.74 \times 10^{-4} ± 2.76 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + 4-DAMP_protect</td>
<td></td>
<td>13.10 ± 163.68</td>
<td>6.00 \times 10^{-4} ± 1.14 \times 10^{-4}</td>
</tr>
<tr>
<td>4</td>
<td>Bethanechol</td>
<td>NA</td>
<td>14.60 ± 52.04</td>
<td>1.00 \times 10^{-4} ± 6.81 \times 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + tropicamide</td>
<td>M₄</td>
<td>10.95 ± 41.45</td>
<td>1.00 \times 10^{-4} ± 6.53 \times 10^{-4}</td>
</tr>
</tbody>
</table>

For each experiment, \( n = 10 \).

* Within a column within an experiment, values with different superscript letters differ significantly (\( P < 0.05 \); comparisons among mAChR antagonists were made only for AF-DX 116 vs 4-DAMP and for methoctramine vs p-F-HHSiD [analysis 2]).

†,‡ Values with the same symbol differ significantly (\( P < 0.05 \)).

Table 1—Mean ± SD values for \( V_{\text{max}} \) and \( EC_{50} \) values for various mAChR antagonists on bethanechol concentration-response curves for basal tone for DL.

Analysis of results of the study reported here revealed that bethanechol has in vitro contractile effects on smooth muscle obtained from the duodenum and jejunum of healthy cows because it induces a significant, concentration-dependent increase in basal tone, \( A_{\text{max}} \), and AUC. Despite the fact that the responsiveness to bethanechol differs among species and anatomic sites, our findings are consistent with the effect of bethanechol described for the stomach and small intestines of horses. In cattle, contractile effects of bethanechol have been reported for the abomasum, ileum, cecum, and pelvic flexure of horses. In contrast, analysis of results of another study on in vitro effects of bethanechol at higher concentrations \( (1 \times 10^{-4} \) to \( 3 \times 10^{-4} \)M) than those used in the study reported here \( (1 \times 10^{-7} \) to \( 1 \times 10^{-6} \)M) indicated no effect of bethanechol on contractility variables in smooth muscle obtained from the proximal portion of the duodenum \( (10 \) cm aboral to the pylorus) in healthy yearling cattle. Bethanechol significantly increases the myoelectric spike rate of the duodenum at locations 5, 10, and 15 cm aboral to the pylorus. In contrast, analysis of results of another study on in vitro effects of bethanechol at higher concentrations \( (1 \times 10^{-4} \) to \( 3 \times 10^{-4} \)M) than those used in the study reported here \( (1 \times 10^{-7} \) to \( 1 \times 10^{-6} \)M) indicated no effect of bethanechol on contractility variables in smooth muscle obtained from the proximal portion of the duodenum \( (10 \) cm aboral to the pylorus) in healthy yearling cattle. Bethanechol significantly increases the myoelectric spike rate of the duodenum at locations 5, 10, and 15 cm aboral to the pylorus. In contrast, analysis of results of another study on in vitro effects of bethanechol at higher concentrations \( (1 \times 10^{-4} \) to \( 3 \times 10^{-4} \)M) than those used in the study reported here \( (1 \times 10^{-7} \) to \( 1 \times 10^{-6} \)M) indicated no effect of bethanechol on contractility variables in smooth muscle obtained from the proximal portion of the duodenum \( (10 \) cm aboral to the pylorus) in healthy yearling cattle.
may reflect true differences between locations; however, it may also have been attributable to forces exerted on the duodenum during the slaughtering and evisceration process because the entire intestine was suspended by the proximal portion of the intestines for several minutes during the slaughtering process, which may have contributed to lower responsiveness and contractility of the duodenum.

Finally, the contractile effects of bethanechol were significantly more pronounced in circular than in longitudinal preparations for mean amplitude and AUC, significantly more pronounced in circular than in longitudinal preparations of the jejunum. Studies,

Table 2—Mean ± SD values for $V_{\text{max}}$ and EC$_{50}$ values for the effects of mAChR antagonists on bethanechol concentration-response curves for AUC for JC.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>mAChR agonist or antagonist</th>
<th>Primary selectivity</th>
<th>$V_{\text{max}}$ (%)</th>
<th>EC$_{50}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bethanechol</td>
<td>NA</td>
<td>25.05 ± 4.09$^a$</td>
<td>1.45 × 10$^{-5}$ ± 3.54 × 10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + AF-DX 116</td>
<td>M$_2$, M$_3$</td>
<td>33.06 ± 19.37$^a$</td>
<td>3.77 × 10$^{-5}$ ± 5.12 × 10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + 4-DAMP</td>
<td>M$_2$, M$_3$</td>
<td>12.39 ± 3.561$^a$</td>
<td>1.00 × 10$^{-6}$ ± 3.94 × 10$^{-6}$</td>
</tr>
<tr>
<td>2</td>
<td>Bethanechol</td>
<td>NA</td>
<td>14.67 ± 4.85$^b$</td>
<td>2.73 × 10$^{-4}$ ± 2.15 × 10$^{-4}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + methoctramine</td>
<td>M$_3$, M$_4$</td>
<td>15.77 ± 11.29$^a$</td>
<td>4.49 × 10$^{-4}$ ± 8.85 × 10$^{-4}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + p-F-HHSiD</td>
<td>M$_1$, M$_4$, M$_5$</td>
<td>16.15 ± 78.34$^a$</td>
<td>4.12 × 10$^{-3}$ ± 4.85 × 10$^{-3}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + atropine</td>
<td>Nonselective</td>
<td>0.5 ± 5.38$^b$</td>
<td>1.00 × 10$^{-4}$ ± 3.64 × 10$^{-4}$</td>
</tr>
<tr>
<td>3</td>
<td>Bethanechol</td>
<td>NA</td>
<td>11.59 ± 2.77$^b$</td>
<td>1.92 × 10$^{-3}$ ± 1.37 × 10$^{-3}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + pirenzepine</td>
<td>M$_1$, M$_4$</td>
<td>15.97 ± 12.76$^b$</td>
<td>4.90 × 10$^{-3}$ ± 8.12 × 10$^{-3}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + AF-DX 116 + 4-DAMP</td>
<td>M$_1$, M$_2$</td>
<td>1.21 ± 1.46$^a$</td>
<td>1.00 × 10$^{-4}$ ± 8.12 × 10$^{-4}$</td>
</tr>
<tr>
<td>4</td>
<td>Bethanechol</td>
<td>NA</td>
<td>15.07 ± 2.52$^b$</td>
<td>1.32 × 10$^{-4}$ ± 3.35 × 10$^{-4}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + tropicamide</td>
<td>M$_1$, M$_4$</td>
<td>15.03 ± 3.77$^b$</td>
<td>2.09 × 10$^{-5}$ ± 1.57 × 10$^{-5}$</td>
</tr>
<tr>
<td></td>
<td>Bethanechol + 4-DAMP$_{\text{Res}}$</td>
<td>M$_3$, M$_4$</td>
<td>4.21 ± 1,928.01$^a$</td>
<td>4.84 × 10$^{-4}$ ± 5.21 × 10$^{-4}$</td>
</tr>
</tbody>
</table>

For each experiment, n = 10. $^a$Represents results relative to predrug values. See Table 1 for remainder of key.

is M$_2$ ≥ M$_3$ > M$_4$ > M$_5$. The antagonist patterns for 4-DAMP and p-F-HHSiD are M$_3$ > M$_2$ ≥ M$_4$ > M$_5$. 13,45,56 Affinity data for tropicamide are sparse, but this receptor antagonist appears to bind primarily to M$_2$. 51,52 We are not aware of any receptor antagonists with high affinity for M$_4$ receptors.

Studies14,44-45 have revealed that > 1 mAChR subtype may be responsible for mediation of cholineric contraction in tissues. Analysis of results of the study reported here indicated that the effect of bethanechol in the intestinal segments of interest was mediated by both M$_4$, and M$_3$, mAChRs, with activation of M$_3$, mAChRs causing stronger increases in contractility traits than for activation of M$_4$, mAChRs, as indicated by use of both sets of receptor antagonists. This finding is consistent with findings in rodents that have revealed a predomi-

1 mAChR subtype functionally involved in mediation of the cholineric contraction induced by bethanechol, experiments were designed that included prior incubation with mAChR antagonists. Concentration of the anticholinergic agent atropine (10$^{-5}$M) was selected on the basis of results of other studies. 41-43 The fact that this concentration of atropine almost completely abolished the effects of bethanechol confirmed that the mAChRs were blocked and corroborated that the effect of bethanechol is mediated via mAChRs. Concentrations of specific mAChR antagonists used in the study were determined from antagonist affinity values reported in other studies, 8,13,44-46 which allowed maximal occupancy of the receptor subtype of interest with minimal occupation of other subtypes. The mAChR antagonists used in the study reported here possess differing selectivity for the various mAChR subtypes, depending on their affinity patterns. Thus, the affinity pattern for pirenzepine $^a$ is M$_3$ > M$_4$ > M$_5$, whereas the pattern for AF-DX 116 $^{13,45,46}$ is M$_3$ > M$_4$ ≥ M$_2$ > M$_5$, and the pattern for methoctramine $^{13,41,45}$ is M$_2$ ≥ M$_3$ > M$_4$ > M$_5$. The antagonist patterns for 4-DAMP and p-F-HHSiD are M$_3$ > M$_2$ ≥ M$_4$ > M$_5$. 13,45,56 Affinity data for tropicamide are sparse, but this receptor antagonist appears to bind primarily to M$_2$. 51,52 We are not aware of any receptor antagonists with high affinity for M$_4$ receptors.

Studies14,44-45 have revealed that > 1 mAChR subtype may be responsible for mediation of cholineric contraction in tissues. Analysis of results of the study reported here indicated that the effect of bethanechol in the intestinal segments of interest was mediated by both M$_4$, and M$_3$, mAChRs, with activation of M$_3$, mAChRs causing stronger increases in contractility traits than for activation of M$_4$, mAChRs, as indicated by use of both sets of receptor antagonists. This finding is consistent with findings in rodents that have revealed a predominant role of M$_4$, mAChRs in the cholineric mediation of smooth muscle contraction, 2,10,11,13 despite the fact that the density of M$_4$, receptors is higher than that of M$_3$, receptors. 13,10 The M$_4$, mAChRs appear to play a role mainly under pathologic conditions (eg, increased sympathetic activity; in knockout mice deficient in M$_4$, receptors; in animals with dysfunctional M$_3$, receptors; and in some disease states, such as rats with experimentally induced diabetes mellitus, intestinal denervation, or bladder denervation). 3,12,45

The predominant role of M$_3$, mAChRs in smooth muscle contraction, compared with that for M$_4$, mAChRs, may be partly explained by differences in the pathways to mediate these effects. Both receptors are linked to G-protein–coupled second messenger systems; however, in contrast to M$_4$, mAChRs that act directly through activation of phospholipase C, M$_3$, mAChRs indirectly mediate contraction via inhibition of the relaxation caused by β-adrenoceptor stimulation, which causes activation of adenylate cyclase. 3,10,56

Binding characteristics of competitive and irreversibly binding receptor antagonists were confirmed in our study because the competitive M$_4$, mAChR antagonist AF-DX 116 caused a visible rightward shift of concentration-response curves, whereas $V_{\text{max}}$ values for
A_max and AUC did not differ from those for concentration-response curves without receptor antagonists. In contrast, the alkylating M_2 mAChR antagonist 4-DAMP caused a significant decrease in V_max for the examined contractility variables, which could not be counteracted by increases in bethanechol concentrations.

Data analysis to compare the effects of M_1 and M_3 mAChR antagonists alone or in combination was complicated by the fact that it was impossible to test all mAChR antagonists in 1 experimental series. The effects of the irreversibly binding M_3 receptor antagonist 4-DAMP alone or in combination could not be investigated consecutively during the same experiment. It has been reported that repeated stimulation of mAChRs up to 6 consecutive times does not affect the responsiveness of smooth muscle preparations. Limiting the duration of experiments to 5 hours and a maximum of 4 concentration-response curves of bethanechol conducted in 1 experimental series allowed us to exclude bias caused by exhaustion of the muscle preparations. However, the experiments had to be conducted in 4 series, which complicated data analysis. Expression of the results for A_max and AUC relative to the corresponding predrug periods and the corresponding bethanechol concentration-response curves allowed us to conduct valid comparisons among receptor antagonists in the various experiments, but this transformation was not possible for basal tone because of the numerous extremely low values at the beginning of the experiments (predrug values).

Use of M_1 and M_3 mAChR antagonists alone or in combination resulted in significantly smaller EC_50 values for the bethanechol concentration-response curves for 4-DAMP, than after prior incubation of muscle preparations with 4-DAMP alone or with a combination of AF-DX 116 and 4-DAMP. The protection assay was designed to eliminate potential unspecific effects of the M_1 mAChR antagonist 4-DAMP on M_3 mAChRs during incubation with 4-DAMP alone. Protection of M_3 mAChRs by use of AF-DX 116 during the incubation period was followed by thorough flushing; thus, the observed effect of 4-DAMP must have been caused through binding of bethanechol to M_1 mAChRs. The fact that the inhibitory effect of the M_3 receptor antagonist 4-DAMP (2 × 10^-6M) without AF-DX 116 to protect the M_3 receptors was significantly greater than in the protection assay indicated that there is unspecific binding of 4-DAMP to the M_3 mAChRs. This observation corroborates the fact that commercially available mAChR antagonists possess limited selectivity for specific receptor subtypes and confirms that their effects cannot be assigned exclusively to 1 mAChR subtype.

Analysis of results of the study reported here suggests that the M_3 and M_4 mAChR subtypes are not involved in mediation of the contractile effect of bethanechol in the locations examined, although M_1 mAChRs have been found in the plexus submucosus and myenteric plexus of the bovine GI tract (from the abomasum to the colon) by use of immunohistochemical analysis. In the ileum of guinea pigs, blot hybridization techniques revealed large amounts of mRNA coding for M_1, a small amount of mRNA coding for M_3, and only traces of mRNA for M_4. In a study of the colon of dogs, M_2 and M_3 mAChR subtypes, but not M_1 and M_4 subtypes, were detected by use of radioligand binding and cDNA hybridization. The choice for the pirenzepine (an M_1 receptor antagonist) concentration used here (10^-5M) was determined on the basis of protocols from other studies in which it was judged that this concentration achieved adequate inhibition of M_1 mAChRs. Therefore, it does not appear that M_3 receptors are involved in mediation of smooth muscle contraction induced by bethanechol. This observation does not preclude a possible role of M_3 mAChRs in the regulation of intestinal motility, but such effects of M_3 receptors would be mediated by other receptor agonists. Our results concerning the role of M_3 receptors in cholinergic mediation of contraction is in accordance with the fact that M_3 receptors were not detected by use of immunohistochemical analysis in the bovine GI tract.

Bethanechol increased contractility variables of smooth muscle specimens obtained from the duodenum and jejunum of healthy cows in a concentration-dependent manner. In general, the contractile effects of bethanechol were more pronounced in jejunal than in duodenal specimens and in circular than in longitudinal preparations. Furthermore, analysis of the results of experiments involving mAChR antagonists indicated that the effect of bethanechol was primarily mediated via M_1 mAChRs and, with a minor role, via M_3 mAChRs. A similar effect of bethanechol has been reported for the abomasum, ileum, and large intestine of cattle in vitro. Thus, analysis of results of the study reported here and other studies suggest that there may be potential beneficial clinical effects from the use of bethanechol as a prokinetic drug for GI motility disorders, such as paralytic ileus, displacement of the abomasum, or cecal dilatation in cattle. Additional studies are warranted to investigate the in vivo effects of bethanechol in cows with the aforementioned diseases.

a. Modified Krebs solution, Dr. E. Graeub AG, Berne, Switzerland.
b. Carbogen, Carbagas, Liebefeld-Bern, Switzerland.
c. Organ baths, ML0186, LSI LETICA, Panlab s.l., Barcelona, Spain.
d. MLT0201, LSI LETICA, Panlab s.l., Barcelona, Spain.
e. ML119, ADInstruments GmbH, Spechbach, Germany.
f. Power Lab, ADInstruments GmbH, Spechbach, Germany.
g. Carbachol, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.
h. Carbamylmüethylcholine chloride, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.
i. AF-DX 116, Tocris, Bristol, UK.
j. 4-DAMP, Tocris, Bristol, UK.
k. Methoctramine tetrahydrochloride, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.
l. P-HHSiD, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.
m. Atropine sulfate salt hydrate, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland.
n. Pirenzepine dihydrochloride, Tocris, Bristol, UK.
o. Tropicamide, Tocris, Bristol, UK.	p. Dimethyl sulfoxide, Dr. Gegg Chemie AG, Stettlen-Deisswil, Switzerland.
q. ChartTM, Power Lab, ADInstruments GmbH, Spechbach, Germany.
s. SYSTAT, SSPS Inc, Chicago, Ill.

References

42. Stengel PW, Cohen HL. Muscarinic receptor knockout mice: role of muscarinic acetylcholine receptors M(2), M(3), and M(4) in carbamylcholine-induced gallbladder contractility. J Pharmacol Exp Ther 2002;301:643–650.


