

**EXCITATORY MUSCARINIC M<sub>1</sub> RECEPTORS MEDIATE IN THE REGULATION OF GUINEA-PIG TERMINAL ILEUM MOTILITY**

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*In vitro experiments were performed on smooth muscle strips cut out longitudinally or circularly from the terminal ileum about 5 cm from the ileo-coecal sphincter.*

*The aim of this study was to determine the functional role of M<sub>1</sub> muscarinic receptor subtype in the regulation of cholinergic contractions of the guinea-pig terminal ileum. Electrical field stimulation (EFS; 16 Hz) produced in vitro contractile responses of the guinea-pig terminal ileum muscle strips (longitudinal and circular muscle layer). Those contractions were inhibited by 1 μM tetrodotoxin (2±2% of the control response) and 1 μM atropine (1±1% of the control response), thus indicating the activation of intrinsic cholinergic nerves. Exogenous ACh (1 μM) induced contractions that were inhibited by 1 μM atropine, but not by 1 μM tetrodotoxin, indicating a direct effect on the smooth muscle. MCN-343, specific agonist of M<sub>1</sub> receptor subtype (5 μM) induced contractions that were inhibited by 1 μM atropine and by 1 μM tetrodotoxin. M<sub>1</sub> receptor antagonist pirenzepine (10 nM) had no effect on ACh – induced contractions, but reduced EFS – induced contractions by 11±3% and MCN-A-343 induced contractions by 17±4%. In conclusion, specific M<sub>1</sub> receptors modulate terminal ileum contractions by regulating the release of ACh from cholinergic nerves. M<sub>1</sub> receptors, present on cholinergic nerves, function as prejunctional facilitatory autoreceptors.*

*Key words: cholinergic receptors, terminal ileum, prejunctional receptors, smooth muscle*

**INTRODUCTION**

Terminal ileum acts almost a different organ from other regions of the gastrointestinal tract. It maintains a high resting tone and a sphincter - like activity. It is provided with a dense adrenergic and cholinergic innervation. Electrical stimulation (EFS) at 30 Hz produced contractions of adrenergic origin (Kažić, 1975) and at 3 Hz of purinergic origin. The majority of contractile responses of the

guinea-pig terminal ileum were cholinergic and a small part of them were alpha-adrenergic, thus the predominance of cholinergic transmission was suggested (Atanassova and Bayguinov, 1991).

Acetylcholine (ACh) is the major excitatory neurotransmitter in the gastrointestinal tract. Intrinsic cholinergic innervation of the gut is very important for the contractility and tone of the gut. There are multiple muscarinic receptor subtypes that have been identified using molecular and pharmacological techniques: M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>, M<sub>4</sub> (Goyal, 1989) and M<sub>5</sub> subtype was identified later on by Reeve *et al.* (1997). The muscarinic receptor subtypes mediating contractions in the small intestine are controversial.

Most tissues, however contain a mixture of receptor subtypes that may have different locations and may serve distinct physiological functions (Eglen *et al.*, 1996). Besides the smooth muscle muscarinic receptors that mediate smooth muscle contractions, cholinergic neurons in many tissues contain presynaptic muscarinic receptors on nerve terminals that serve to limit ("inhibitory autoreceptor") or enhance ("excitatory autoreceptors") the release of ACh from cholinergic nerves (Goyal, 1989). The ACh released from these intrinsic nerves will act on smooth muscles. Blockade of M<sub>1</sub> receptors enhanced guinea-pig intestinal peristalsis (Schowor and Kilibinger, 1988). Studies on the lower oesophageal sphincter of the opossum (Gielbert *et al.*, 1984) have also demonstrated that the activation of neuronal M<sub>1</sub> receptors causes relaxation of smooth muscles. Inhibition of the motility was due to the activation of inhibitory presynaptic autoreceptors. M<sub>1</sub> receptors were found on cholinergic nerves in guinea-pig gallbladder and function as prejunctional facilitatory autoreceptors (Parkman *et al.*, 1999).

The cholinergic system exerts an excitatory influence on pendular movements on rabbit distal colon, predominantly through activation of presynaptic M<sub>1</sub> muscarinic receptors (Radenovic *et al.*, 1998).

The aim of this study was to determine the functional role of M<sub>1</sub> receptor subtype in the regulation of terminal ileum cholinergic contractions.

#### MATERIAL AND METHODS

Terminal segments of the ileum were dissected from guinea-pigs weighting 300-600 g. which previously have been stunned and bled out.

##### *Contractile responses of the longitudinal muscle layer*

Segments of 2-3 cm were dissected and placed in an organ bath containing Tyrode's solution of the following composition: NaCl 137 mM/L, KCl 2.7 mM/L, NaHCO<sub>3</sub> 11.9 mM/L, CaCl<sub>2</sub> 1.82 mM/L, MgCl 0.11 mM/L and glucose 5.56 mM/L. This solution was bubbled with pure oxygen and kept at a constant temperature of 36°C. Contractions and tonic changes were recorded isotonically via a transducer (Beckman) and recorded on a recorder (Beckman).

##### *Contractile responses of the circular muscle layer*

A segment of the terminal ileum with the adjacent mesentery 1.0-1.5 cm in length was taken and suspended in an organ bath containing Tyrode's solution.

The segment was secured at the bottom of the bath by a horizontal glass passed through the lumen as described by Costa and Furness (1979). The mechanical activity of the circular muscle was recorded by means of a heart clip attached to the adjacent mesentery and connected via a cotton thread to an isometric measuring system (Beckman).

Electrical field stimulation (EFS) was performed by coaxially placed electrodes with trains of pulses delivered from an electronic stimulator (Grass 58). The parameters of the electrical stimulation were as follows: 16 Hz (1-30 Hz), pulse duration 0.2 m/sec, voltage 40-80 V. The following compounds were used: ACh chloride; specific agonist of M<sub>1</sub> muscarinic receptors MCN-A-343; hexamethonium chloride; the nonspecific muscarinic antagonist atropine sulfate; specific M<sub>1</sub> muscarinic receptor subtype antagonist - pirenzepine.

Data are expressed as means  $\pm$ SE of results obtained from 6-8 experiments. Differences between mean values were compared by Student's *t*-test. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

Electrical field stimulation (EFS) of the guinea-pig terminal ileum produced a contractile response of both the longitudinal and the circular muscle layers. Contractile responses were frequency-dependent (from 2 Hz to 30 Hz;  $p < 0.01$ ). All responses were inhibited by 1  $\mu$ M tetrodotoxin ( $2 \pm 2\%$  of control;  $p < 0.01$ ), but not by 100  $\mu$ M hexamethonium ( $102 \pm 3\%$  of control response). Different EFS frequencies showed different sensitivity to 1  $\mu$ M atropine. The contractile response to low (1-3 Hz) and high (30 Hz) frequencies was reduced ( $33 \pm 4\%$  and  $48 \pm 4\%$  of the control response, respectively). This reduction in response was, compared to the control, significant ( $p < 0.01$ ) at both frequencies. Response to 16 Hz was completely inhibited by 1  $\mu$ M atropine ( $1 \pm 1\%$  of the control response;  $p < 0.01$ ) (Table 1).

Table 1. Influence of tetrodotoxine, hexamethonium and atropine on EFS effects

Frequency	30 Hz	16 Hz	2 Hz
Atropine	$48 \pm 2 \%$	$1 \pm 1 \%$	$33 \pm 4 \%$
Tetrodotoxine	$2 \pm 2 \%$	$2 \pm 2 \%$	$2 \pm 2 \%$
Hexamethonium	$102 \pm 3 \%$	$102 \pm 3 \%$	$102 \pm 3 \%$

Data are presented as percent of control response and are reported as means  $\pm$  SE, EFS (electrical field stimulation)

Effects of EFS (from 3 Hz to 30 Hz) were voltage-dependent (40-80 V) too;  $p < 0.001$ .

Motor responses were elicited by ACh, as well. Responses were dose-dependent;  $p < 0.01$ ;  $ED_{50} = 0.45 \mu$ M for the longitudinal muscle layer,  $ED_{50} = 0.60 \mu$ M for the circular muscle layer. The response to ACh was submaximal and

reproducible: the second ACh induced contraction was  $103 \pm 3\%$  of the first administration. Atropine ( $1 \mu\text{M}$ ) completely inhibited the response to ACh (Table 2).

Table 2. ED<sub>50</sub> ( $\mu\text{M}$ ) of some cholinomimetics and their relative potency on terminal ileum

Agonist	ED <sub>50</sub> ( $\mu\text{M}$ )	Relative potency
Longitudinal layer		
ACh	0.457	1.00
MCN-A-343	1.12	0.52
Circular layer		
ACh	0.602	1.00
MCN-A-343	3.60	0.16

ED<sub>50</sub> ( $\mu\text{M}$ ) was calculated from the dose-response curves. Relative potency of MCN-A-343 was calculated as ED<sub>50</sub> ( $\mu\text{M}$ ) of MCN-A-343/ ED<sub>50</sub> ( $\mu\text{M}$ ) of ACh on longitudinal and circular muscle layer.

MCN-A-343, specific agonist of M<sub>1</sub> receptors elected a dose-dependent motor response of the longitudinal and circular muscle layers of the guinea-pig terminal ileum,  $p < 0.01$ .

For the longitudinal muscle layer ED<sub>50</sub> was  $1.1 \mu\text{M}$  and  $3.60 \mu\text{M}$  for the circular muscle layer. MCN-A-343 ( $5 \mu\text{M}$ ) responses were sub maximal and reproducible: the second MCN-A-343 induced contraction was  $104 \pm 5\%$  of the first administration (Table 2). Relative potency of MCN-A-343 (compared to ACh) was 0.52 on longitudinal muscle layer and 0.16 on circular muscle layer (Table 2). Atropine ( $1 \mu\text{M}$ ) completely inhibited the muscle response to MCN-A-343 ( $p < 0.01$ ).

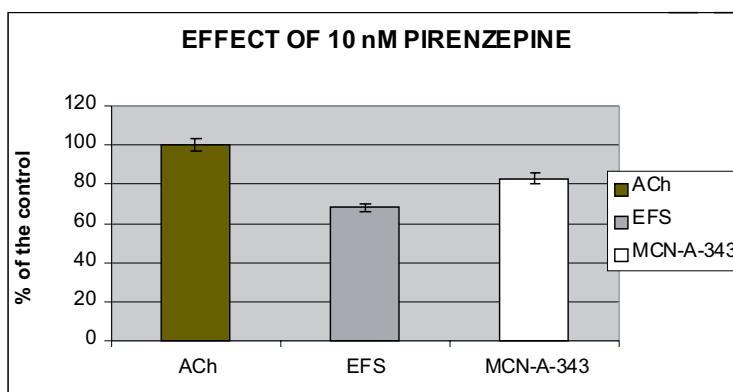


Figure 1. Effects of low concentration (10 nM) of M<sub>1</sub> muscarinic antagonist pirenzepine on ACh induced, EFS induced and MCN-A-343 induced contractions. Results are expressed as means  $\pm$  SE from 6 muscle strips

The M<sub>1</sub> selective antagonist pirenzepine in a low dose (10 nM) had no effect on ACh-induced contractions, but significantly inhibited EFS (16 Hz)-induced contractions. Pirenzepine inhibited EFS-induced contractions preferentially at higher frequencies (16 Hz) than at low frequencies (2 Hz) and very high frequencies (30 Hz). Pirenzepine (10 nM) had no effect on ACh – induced contractions, but reduced EFS – induced contractions by  $11 \pm 3\%$ ; ( $p < 0.01$ ) and MCN-A-343 induced contractions by  $17 \pm 4\%$  ( $p < 0.01$ ) (Fig. 1). Higher doses of pirenzepine (1  $\mu\text{M}$ ) inhibited the contractile responses to ACh, EFS and MCN-A-343 (Fig 2). ACh induced contractions were inhibited by  $31 \pm 4\%$ , EFS induced contractions were inhibited by  $44 \pm 7\%$  and MCN-A-343 by  $52 \pm 6\%$ ; ( $p < 0.01$ ) (Fig. 2).

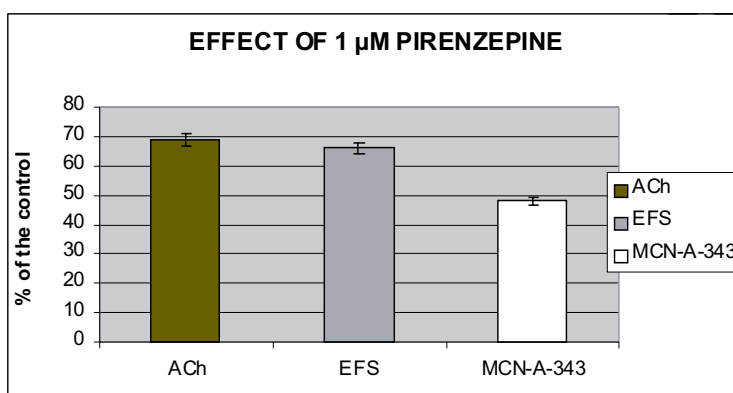


Figure 2. Effects of high concentration (1  $\mu\text{M}$ ) of M<sub>1</sub> muscarinic antagonist pirenzepine on ACh induced, EFS induced and MCN-A-343 induced contractions. Results are expressed as means  $\pm$  SE from 6 muscle strips

## DISCUSSION

Most tissues contain a mixture of receptor subtypes that have different locations and may serve distinct functions (Eglen *et al.*, 1996). Muscarinic receptors in the gastrointestinal tract are present on enteric neurons (Takeuchi *et al.*, 2005), presynaptic and prejunctional axonal endings, intramural endocrine cells, as well as directly on effector cells such as smooth muscle and glandular and epithelial cells (Goyal, 1988). Neural M<sub>1</sub> stimulatory receptors are present on myenteric and submucous neurons (Goyal, 1988).

The terminal ileum differs from other parts of the ileum, as it has a sphincter-like function. EFS elected mainly excitatory responses of the muscle strips of this region. The majority of this responses were cholinergic and a small part of them alpha-adrenergic and serotonergic, thus the predominance of cholinergic transmission in the contractile responses of the terminal ileum was suggested by Atanassova and Bayguinov (1991).

EFS in rabbit anoccygeus muscle elected contractile responses too. EFS resulted in a release of noradrenaline electing monophasic contractions due to

stimulation of postjunctional alpha1-adrenoreceptors. The selective muscarinic antagonists caused concentration-related inhibition of neurogenic responses indicating a regulatory role of muscarinic receptors (Lamberecht and Mutschler, 1999).

The distribution of muscarinic receptor subtypes throughout the gastrointestinal tract is rather complex and exhibits regional, as well as species differences (Matsui *et al.*, 2004). Muscarinic M<sub>3</sub> type appears to be mainly located postsynaptically in the smooth muscle (Hanack and Pfeifer, 1990). Since both M<sub>1</sub> and M<sub>2</sub> subtypes may be found at neuronal structures they could regulate motor activity (Gonzales *et al.*, 2004). The excitatory M<sub>1</sub> receptors are found on neuronal bodies in the myenteric plexus.

There are many contradictory data about the role of M<sub>1</sub> subtype of muscarinic receptors in mediating the cholinergic responses of different parts of the gastrointestinal tract. In some parts they have inhibitory, but in other parts they display an excitatory effect. On the lower oesophageal sphincter of the opossum (Gielbert *et al.*, 1984) EFS (16 Hz) produced contractile responses of the guinea-pig gallbladder muscle strips *in vitro* that were reduced by pirenzepine, which is an M<sub>1</sub> receptor antagonist (Parkman *et al.*, 1999), so it was concluded that M<sub>1</sub> receptors are in this region excitatory.

Neuronal M<sub>1</sub> receptors caused inhibition of the small intestinal peristalsis in guinea-pig isolated small intestine, so they seemed to have an inhibitory role in the reflex function of the small intestine (Schworer and Kilbinger, 1998). M<sub>1</sub> receptors have an inhibitory role in regulating muscle tone of the lower oesophageal sphincter of the opossum (Gielbert *et al.*, 1984). M<sub>1</sub> receptors regulate spontaneous motor activity in rabbit and cat colon (Goyal, 1988).

Both a non-selective muscarinic antagonist atropine and selective, predominantly M<sub>1</sub> receptor antagonist pirenzepine reduced the effects of EFS and cholinomimetics.

In our experiment, EFS of guinea-pig terminal ileum (longitudinal and circular muscle layer) produces a contractile response that was inhibited by tetrodotoxin. The EFS-induced contractions are also inhibited by atropine suggesting that the contractile response of guinea-pig terminal ileum originates from the activation of intrinsic cholinergic nerves. The M<sub>1</sub> selective receptor antagonist pirenzepine in low doses (10 nM) had no effect on ACh-induced contractions but significantly inhibited EFS-induced contractions. This suggests that M<sub>1</sub> receptors are located on cholinergic nerves and function normally when activated to increase the release ACh from nerves. They function as presynaptic excitatory autoreceptors. Pirenzepine inhibited EFS-induced contractions preferentially at higher frequencies than at lower frequencies. Thus, in the terminal ileum, M<sub>1</sub> presynaptic facilitory autoreceptor is activated with higher frequency stimulation (16 Hz). Recent molecular studies showed that ACh acting at nAChRs and M<sub>1</sub> muscarinic receptors is an important excitatory neurotransmitter in the enteric nervous system. M<sub>1</sub> muscarinic receptors are G protein-coupled receptors that link to activation of phospholipase C and generation of inositol triphosphate and diacylglycerol (Brown and Gallian, 2003).

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## EKSCITATORNI MUSKARINSKI M<sub>1</sub> RECEPTORI POSREDUJU U REGULACIJI MOTILITETA TERMINALNOG ILEUMA ZAMORČETA

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### SADRŽAJ

Cilj ovog rada je bio da se odredi funkcionalna uloga M<sub>1</sub> podtipa muskarinskih receptora u regulaciji holinergičkim putem izazvanih kontrakcija terminalnog ileuma zamorčeta. Eksperimenti su izvedeni na isečcima longitudinalnog i cirkularnog mišićnog sloja terminalnog ileuma zamorčeta u predelu udaljenom oko 5 cm od ileocekalnog spoja. Stimulacija električnim poljem (EFS; 16 Hz) izaziva kontraktilne odgovore terminalnog ileuma zamorčeta (longitudinalnog i cirkularnog mišićnog sloja) u uslovima *in vitro*. Ove kontrakcije su inhibirane sa 1 μM tetrodotoksina (2±2% od kontrolne vrednosti) i 1 μM atropina (1±1% od kontrolne vrednosti) što ukazuje na aktivaciju unutrašnjih holinergičkih nerava. Egzogeni ACh (1 μM) izaziva kontrakcije koje su inhibirane sa 1 μM atropina, ali ne i 1 μM tetrodotoksina, što ukazuje na direktne efekte na glatku muskulaturu. MCN-A-343, specifični agonista M<sub>1</sub> podtipa receptora (5 μM) izaziva kontrakcije koje su inhibirane sa 1 μM atropina i sa 1 μM tetrodotoksina. Antagonist M<sub>1</sub> receptora, pirenzepin (10 nM), ne deluje na kontrakcije izazvane sa ACh, ali redukuje kontrakcije izazvane sa EFS za 11±3% i kontrakcije izazvane sa MCN-A-343 za 17±4%.

Na osnovu ovih rezultata se može zaključiti da specifični M<sub>1</sub> receptori moduliraju kontrakcije terminalnog ileuma regulišući oslobađanje ACh sa holinergičkih nerava. M<sub>1</sub> receptori su prisutni na holinergičkim nervima i funkcionišu kao presinaptički facilitatorni autoreceptori.