

• BASIC RESEARCH •

# Mechanisms mediating cholinergic antral circular smooth muscle contraction in rats

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## Abstract

**AIM:** To investigate the pathway (s) mediating rat antral circular smooth muscle contractile responses to the cholinomimetic agent, bethanechol and the subtypes of muscarinic receptors mediating the cholinergic contraction.

**METHODS:** Circular smooth muscle strips from the antrum of Sprague-Dawley rats were mounted in muscle baths in Krebs buffer. Isometric tension was recorded. Cumulative concentration-response curves were obtained for (+)-cis-dioxolane (cD), a nonspecific muscarinic agonist, at  $10^{-8}$ - $10^{-4}$  mol/L, in the presence of tetrodotoxin (TTX,  $10^{-7}$  mol/L). Results were normalized to cross sectional area. A repeat concentration-response curve was obtained after incubation of the muscle for 90 min with antagonists for M1 (pirenzepine), M2 (methoctramine) and M3 (darifenacin) muscarinic receptor subtypes. The sensitivity to PTX was tested by the ip injection of 100 mg/kg of PTX 5 d before the experiment. The antral circular smooth muscles were removed from PTX-treated and non-treated rats as strips and dispersed smooth muscle cells to identify whether PTX-linked pathway mediated the contractility to bethanechol.

**RESULTS:** A dose-dependent contractile response observed with bethanechol, was not affected by TTX. The pretreatment of rats with pertussis toxin decreased the contraction induced by bethanechol. Lack of calcium as well as the presence of the L-type calcium channel blocker, nifedipine, also inhibited the cholinergic contraction, with a reduction in response from  $2.5 \pm 0.4$  g/mm<sup>2</sup> to  $1.2 \pm 0.4$  g/mm<sup>2</sup> ( $P < 0.05$ ). The dose-response curves were shifted to the right by muscarinic antagonists in the following order of affinity: darifenacin (M<sub>3</sub>) > methoctramine (M<sub>2</sub>) > pirenzepine (M<sub>1</sub>).

**CONCLUSION:** The muscarinic receptors-dependent contraction of rat antral circular smooth muscles was linked to the signal transduction pathway(s) involving pertussis-toxin sensitive GTP-binding proteins and to extracellular calcium via L-type voltage gated calcium channels. The presence of the residual contractile response after the treatment with nifedipine, suggests that an additional pathway could mediate the cholinergic contraction. The involvement of more than one muscarinic receptor (functionally predominant type 3 over type 2) also suggests more than one pathway mediating the cholinergic contraction in rat antrum.

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## INTRODUCTION

The mechanisms involved in the regulation of cholinergic contraction of intestinal smooth muscle are complex and not fully understood, despite the important role of the cholinergic system in the physiology of gastric emptying, and pathophysiology of several motility disorders. Cholinergic agonists activate muscarinic receptors which transduce cholinergic signals by activating G proteins<sup>[1,2]</sup>. Different signal transduction pathways in different species, as well as different pathways for the circular and longitudinal layers of intestinal smooth muscle have been reported<sup>[3-15]</sup>.

Specific muscarinic receptors are abundantly present in the smooth muscles of gastrointestinal tract<sup>[16-22]</sup>. Muscarinic receptor subtypes have shown a G-protein coupling specificity, however the published data are inconsistent. In some studies M<sub>1</sub>, M<sub>3</sub>, M<sub>5</sub> receptor subtypes were preferentially coupled to the Gq/11 protein class, the M<sub>2</sub> and M<sub>4</sub> receptors were linked to the PTX-sensitive Gi/Go proteins<sup>[23-25]</sup>. Whereas in other studies muscarinic M<sub>2</sub> receptors were insensitive to pertussis toxin<sup>[26]</sup>.

Relatively few references are published characterizing rat stomach muscarinic receptor subtypes, again with conflicting results. Prevalence of M<sub>3</sub><sup>[13,22,27]</sup>, or of M<sub>1</sub><sup>[28]</sup>, or M<sub>2</sub> receptors<sup>[29-33]</sup> has been reported. Which muscarinic receptor is more functionally important in the cholinergic contraction of antral circular muscle has not been determined.

The aim of this study was to examine the signal transduction pathway (s) mediating rat antral smooth muscle cholinergic contraction to fill the existing knowledge gaps: (1) what type of calcium channel was involved and was there a dependence on extracellular Ca<sup>2+</sup> influx; (2) whether PTX-sensitive- or PTX-insensitive-G proteins coupled to muscarinic receptors were involved; and (3) what subtypes of specific muscarinic receptors were functionally involved. Determining the physiology of the pathway (s) mediating cholinergic contraction of the antrum should be helpful in understanding the functional motility changes described in models of disease conditions, such as diabetes<sup>[34-36]</sup>. Preliminary accounts of some of these observations have been published in abstract form<sup>[37,38]</sup>.

## MATERIALS AND METHODS

### Animals

Young adult male Sprague-Dawley rats (Charles River Breeding Laboratories), weighing 200-450 g were used. Animals were anesthetized by ip injection of sodium pentobarbital (30-65 mg/kg). Anesthesia was given immediately before the tissue removal to avoid the effect of anesthesia on the contractile properties of the tissue. The abdomen was explored through midline incision and the stomach was removed. After the tissue was removed, animals were euthanized by injection of an overdose of pentobarbital. All our studies were approved by the Institutional Animal Care and Use Committee of the PSU College of Medicine.

### Smooth muscle strip bath preparation<sup>[39]</sup>

The antrum tissue was pinned in a dissecting dish in oxygenated Krebs solution, mucosa was gently removed by scraping, and strips were cut in the circular muscle orientation. The muscle was oxygenated in Krebs physiological buffer containing (in mmol/L) 130 Na, 4.7 K, 2.5 Ca, 1.0 Mg<sub>2</sub>, 140.7 Cl, 19 HCO<sub>3</sub>, 1.0 PO<sub>4</sub> and 10 glucose at 37 °C. The strips were sutured at one end to a glass rod and at the other end to an inelastic wire. The tissue, the rod and a wire were placed in a 10-mL double walled glass chamber with a constant temperature 37 °C. Glass surfaces were silicone coated with Sigmacote to prevent binding of the peptides. The chambers were filled with 5 mL of Krebs buffer and gassed with 95:5 mixture of O<sub>2</sub>/CO<sub>2</sub>. The free end of wire was attached to an isometric force transducer (Grass Instrument Co) and recordings were made on a multichannel rectilinear dynograph recorder (Beckman Instruments). The strips were allowed to equilibrate for 1 h. Tissues were stretched to an initial length (L<sub>i</sub>) from which any additional stretch resulted in an increase in tension. The isometric tension response to bethanechol (10<sup>-4</sup> mol/L) was noted. The strips were rinsed and the length increased by 1 mm increment until the maximum response to bethanechol (10<sup>-4</sup> mol/L) was recorded. This length was labeled L<sub>o</sub>. All subsequent studies were conducted at this length. Drugs were added to the tissue bath and the peak response within 8 min was compared with the maximum tension recorded during 5 min before addition of drugs. Antagonists were added 1 min before the addition of agonist. The peptidase inhibitors bestatin and phosphoramidon (both at 10<sup>-6</sup> mol/L) were added at least 5 min before the agonist addition. The response was calculated as the change in the maximal force of contraction in g of tension normalized to the cross sectional area which was calculated as: cross section (mm<sup>2</sup>) = weight (g)/specific density × length (mm), where the specific density of muscle tissue = 1.056 (g/mm<sup>3</sup>).

### Dispersed single muscle cell preparation<sup>[40,41]</sup>

Smooth muscle cells were isolated from the circular muscle layer of the antrum. The mucosa was removed by dissection and the longitudinal muscle layer (with enteric plexus ganglia) was removed in strips using a Stadie-Riggs tissue slicer (Thomas TM). The circular muscle layer was minced and incubated for 45 min twice in Hepes buffer containing collagenase (CLS type II, Worthington) and 0.1 g/L soybean trypsin inhibitor at 31 °C. Partially digested strips were washed with enzyme-free Hanks' medium. Muscle cells were allowed to disperse spontaneously under the gentle force of bubbling 950 mL/L O<sub>2</sub>+50 mL/L CO<sub>2</sub> for 30 min (no agitation), and then filtered through 500 µmol/L Nitinol mesh, to the culture media. Viability was checked by trypan blue exclusion test. Aliquots of cells were added to solutions containing the agonists at room temperature. The reaction was stopped after 30 min, by addition of acrolein to the final concentration of 1%. Aliquots were sealed under coverslips. Computerized image analysis (NIH Image 1.62) was used for quantification. A scale slide was used for reference. The length of single muscle cells was measured (at least 50 cells per concentration).

### Contractile response of antrum smooth muscle strips to cholinergic agonist

To determine the effect of bethanechol on antrum circular smooth muscle contraction, muscle strips were exposed to the increasing doses of muscarinic agonist, bethanechol, at the concentrations of 10<sup>-4</sup> to 10<sup>-7</sup> mol/L in an organ bath.

### Characteristics of effect of extracellular Ca<sup>2+</sup> and type of calcium channel involved

To determine whether cholinergic contraction depended on the influx of extracellular Ca<sup>2+</sup>, the response to bethanechol chloride in the physiological Krebs buffer was tested, then the buffer was changed into Ca-free buffer (in mmol/L: NaCl 132.5; KCl 4.

7; MgCl<sub>2</sub> 1.0; NaH<sub>2</sub>PO<sub>4</sub> 1.2; NaHCO<sub>3</sub> 20.0; D-glucose 10; EGTA (50 µmol/L), and contraction was subsequently recorded after 5 and 10 min.

To characterize the type of calcium channel involved in the cholinergic contraction, nifedipine (an L-type calcium channel antagonist) was used. Nifedipine was dissolved in ethanol and added to the physiological Krebs buffer at the concentration of 10<sup>-5</sup> mol/L<sup>[42]</sup> and contraction was recorded. Statistical significance of the difference between contraction in the presence and absence of nifedipine was calculated by paired *t*-test, the results were considered statistically significant at *P* ≤ 0.05.

### Treatment with pertussis toxin (PTX)

In order to determine whether PTX-sensitive pathway was involved in cholinergic contraction, strips and dispersed muscle cells (myocytes) isolated from the antrum of PTX-pretreated and non-pretreated animals were compared.

Rats were injected with 100 mg/kg of PTX (dissolved in saline) intraperitoneally 5 d before the study<sup>[43]</sup>. Muscle strips from PTX-treated and control rats in the tissue bath were exposed to cholinergic agonist, bethanechol, at the concentration of 10<sup>-4</sup> to 10<sup>-6</sup> mol/L. Statistical significance of the difference between the contraction of the muscle from PTX-pretreated and non-treated rats was defined by non-paired *t*-test, the results were considered statistically significant at *P* ≤ 0.05.

The changes in the pattern of contraction of muscle cells in dispersed cell suspension were also measured (detailed description in the "dispersed muscle cell preparation" section of Materials & Methods). Two concentrations of bethanechol (10<sup>-7</sup> and 10<sup>-8</sup> mol/L) were added to the cell suspensions in the tubes in the physiological buffer. Their contractions were measured as the percentage of the control cell diastolic length<sup>[44]</sup>. The mean lengths of cells from control rats were compared to those of the cells from PTX-treated animals. Results were presented as mean ± SE. Statistical significance of the difference was calculated by the paired *t*-test, the results were considered statistically significant at *P* ≤ 0.05.

### Characterization of muscarinic receptor subtypes involved

For the characterization of muscarinic receptor subtypes involved in cholinergic contraction we used a non-selective muscarinic agonist, (+)-cis-Dioxolane<sup>[45,46]</sup> and relatively specific receptor subtype antagonists.

The conditions of organ bath were described above in the "Smooth muscle strip bath preparation" section of Materials and Methods. At the start of the experimental protocol, the viability of each tissue was assessed by determining the contractile response to bethanechol (10<sup>-4</sup> mol/L). After washed, tissues were re-equilibrated for 10 min and allowed to regain baseline tension. Cumulative concentration-effect curves of (+)-cis-Dioxolane, (10<sup>-8</sup> to 3 × 10<sup>-5</sup> mol/L) were constructed for each tissue. Tissues were then equilibrated in either the absence (control) or presence of the antagonist for 90 min. Subsequently, a second concentration-effect curve to (+)-cis-Dioxolane was constructed. Smooth muscle strips were incubated with increasing concentrations of antagonists demonstrating a relative specificity for M<sub>1</sub>, M<sub>2</sub> or M<sub>3</sub> muscarinic receptor subtypes (pirenzepine, methoctramine and darifenacin, respectively). Each antral smooth muscle strip was exposed to only one concentration of antagonists and incubated for 90 min at 37 °C, with a fresh antagonist added to the medium every 30 min<sup>[47,48]</sup>.

The EC<sub>50</sub> values for muscarinic antagonists were obtained (i.e. antagonist concentration resulting in 50% of inhibition of the contraction induced by cholinergic agonist, (+)-cis-Dioxolane (10<sup>-6</sup> mol/L).

### Drugs

Tetrodotoxin (TTX), sigmacote, neurokinin A (NKA), nifedipine,

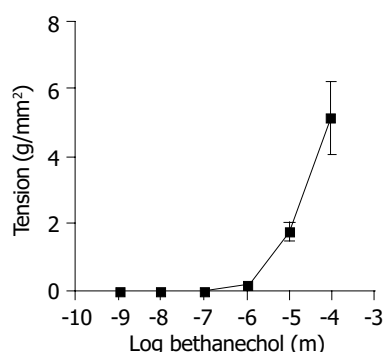
papain, peptidase inhibitors bestatin and phosphoramidon, soybean trypsin inhibitor, acrolein and pirenzepine (predominantly  $M_1$  muscarinic receptor antagonist), were from Sigma, St. Louis, MO.

(+)-cis-dioxolane (cholinergic agonist) and methocramine (predominantly  $M_2$  muscarinic receptor antagonist) were purchased from RBI Inc., Natick, MA. PTX was purchased from List Biological Labs, Inc., Campbell, CA. Bethanechol chloride was purchased from Merck, West Point, PA and collagenase (CLS type II) from Worthington, PA. Darifenacin (predominantly  $M_3$  muscarinic receptor antagonist) was a generous gift from Pfizer Ltd, Sandwich, Kent, GB.

## RESULTS

### Dose-response curve to cholinergic agonist

A contractile dose-response was observed, when the antral circular smooth muscle strips were exposed to the increasing doses of muscarinic agonist, bethanechol, at the concentrations of  $10^{-4}$  to  $10^{-7}$  mol/L in an organ bath. A significant increase of the tension over the baseline was observed at the bethanechol concentrations of  $10^{-4}$  to  $10^{-6}$  mol/L (Figure 1,  $n = 4$ ).



**Figure 1** Effect of bethanechol on smooth muscle contraction. Antrum circular smooth muscle strips were incubated with increasing concentrations of bethanechol ( $10^{-4}$  mol/L to  $10^{-7}$  mol/L). The vertical axis represents the developed tension (in grams per  $mm^2$ ). Bethanechol significantly increased circular muscle tension ( $P < 0.05$ ; paired  $t$ -test). Each data point represents mean  $\pm$  SE,  $n = 4$ .

### Effect of tetrodotoxin (TTX)

Antrum circular smooth muscle strips were exposed to bethanechol at the concentration of  $10^{-5}$  mol/L, in a buffer containing tetrodotoxin ( $10^{-5}$  mol/L), added prior to bethanechol to the organ bath. The cholinergic contraction was not affected by tetrodotoxin, neuronal toxin, with the contraction at  $94.9 \pm 3.2\%$  of control.

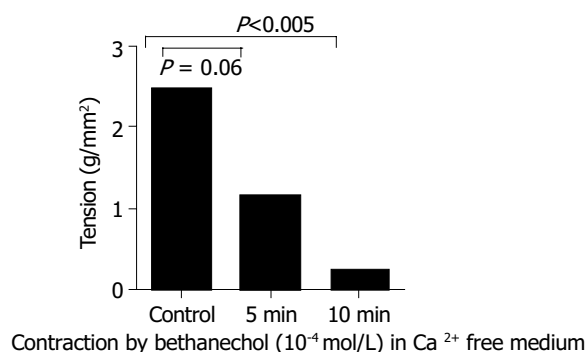
The lack of inhibition of the contraction by TTX confirmed that a cholinergic agonist could induce contraction via muscarinic receptors present at the smooth muscle, without participation of neuronal factor.

### Effect of calcium depletion on cholinergic contraction and characterization of a calcium channel subtype involved

**Calcium-free buffer** Incubation in calcium-free buffer diminished the antral contractile response to bethanechol ( $10^{-4}$  mol/L), with a reduction in response from  $2.5 \pm 0.4$  g/ $mm^2$  to  $1.2 \pm 0.4$  g/ $mm^2$  ( $P < 0.05$ ) after 5 min of incubation in calcium-free buffer. After 10 min of incubation in  $Ca^{2+}$ -free buffer contractile activity was almost abolished (Figure 2).

Full recovery of the contraction was seen after return of the  $Ca^{2+}$  to the tissue bath medium, indicating that there was no damage to the cell resulting in a decreased contraction. These results showed that cholinergic contraction was dependent on the presence of extracellular calcium, a receptor-operated  $Ca^{2+}$  or voltage-sensitive  $Ca^{2+}$  channels.

**Effect of calcium channel blocker** The L-type calcium channel blocker, nifedipine at a concentration of  $10^{-5}$  mol/L inhibited the cholinergic contraction response of antrum to the bethanechol, at the concentration of  $10^{-4}$  mol/L from  $4.02 \pm 0.9$  to  $0.49 \pm 0.18$  g/ $mm^2$  ( $P < 0.05$ ), indicating that the L-calcium channel could mediate this contraction.

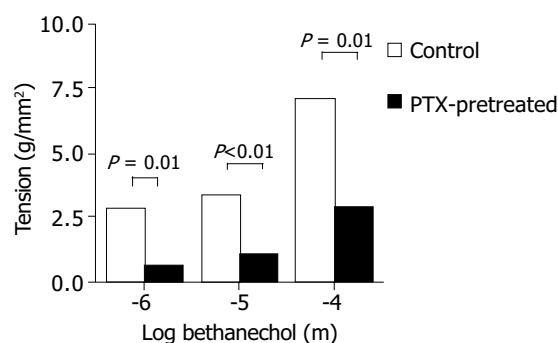


**Figure 2** Effect of depletion of  $Ca^{2+}$  from medium on the contraction of antral circular smooth muscle strips to bethanechol ( $10^{-4}$  mol/L).  $Ca^{2+}$  depletion caused a significant decrease in the muscle tension; after 5 min incubation ( $P < 0.06$ , paired  $t$ -test); and after 10 min incubation ( $P < 0.005$ , paired  $t$ -test) compared to the base contraction induced by bethanechol before  $Ca^{2+}$  depletion. Each data point represents mean  $\pm$  SE,  $n = 3$ .

### Identification of G-protein-linked signal transduction pathway by PTX

The response to bethanechol of antral circular muscle strips and dispersed smooth muscle cells from PTX-pretreated and control rats was compared (see Methods).

**Smooth muscle strips** The contractile response to bethanechol of the muscle strips from the PTX-pretreated animals was significantly lower than that of the muscle strip from the non-treated control rats at all concentrations,  $10^{-6}$  to  $10^{-4}$  mol/L (Figure 3,  $P \leq 0.01$ ).



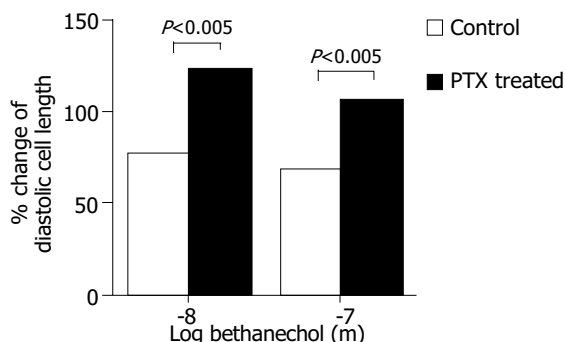
**Figure 3** Effect of PTX on antrum circular smooth muscle strips contraction to bethanechol ( $10^{-4}$  mol/L to  $10^{-6}$  mol/L). PTX significantly inhibited the contractile activity of the smooth muscle ( $P \leq 0.01$ ; paired  $t$ -test) compared to control rats. Each point represents mean  $\pm$  SE,  $n = 7$ ; dotted bars represent control animals; solid bars-PTX-pretreated animals.

The inhibiting effect of PTX on the contraction implied that cholinergic agonist was activating a PTX-sensitive pathway.

A low level of residual contractile activity was observed in the contraction of PTX-treated muscle strips suggesting either that there was a small PTX-insensitive fraction involved, or that the dose of PTX used was insufficient to completely inhibit PTX-sensitive pathways.

**Dispersed antral smooth muscle cells** When dispersed myocytes from PTX-treated rats and controls were compared, cells from PTX-pretreated rats showed significantly less contraction to

bethanechol than the cells isolated from the rats non-treated with PTX, at both bethanechol concentrations,  $10^{-8}$  and  $10^{-7}$  mol/L (Figure 4,  $P \leq 0.005$ ). The inhibiting effect of PTX on the dispersed myocytes contraction, confirmed the smooth muscle strip data and suggested that the cholinergic agonist involved an occupation of specific muscarinic receptors coupled to PTX-sensitive mediated pathway.

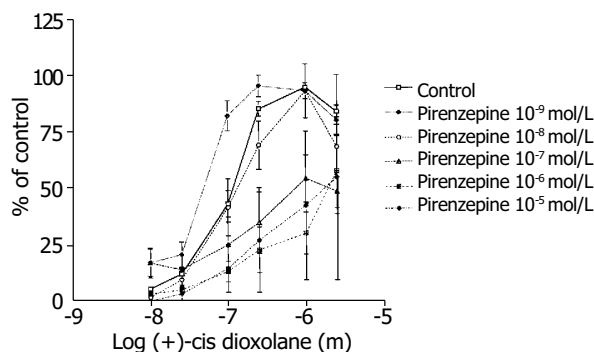


**Figure 4** Effect of PTX on dispersed antral circular smooth muscle myocytes from PTX-pretreated rats contraction to bethanechol ( $10^{-7}$  mol/L and  $10^{-8}$  mol/L). Myocytes from PTX-treated rats contracted less than myocytes from control rats. Each point represents mean  $\pm$  SE; of the percentage of the control cells diastolic length (no bethanechol). At least 50 myocytes per point were calculated,  $P < 0.005$ ;  $n = 7$  and 11. Dotted bars represent control animals; solid bars PTX-pretreated animals.

**Characterization of the type of muscarinic receptor subtypes involved in contraction to cholinergic agonist** To define pharmacologically the muscarinic receptors involved in the cholinergic contraction, specific muscarinic receptor antagonists were used in the presence of tetrodotoxin ( $10^{-7}$  mol/L).

For this functional muscarinic receptor study we chose a non-selective cholinergic agonist, (+)-cis-Dioxolane (see Methods), instead of bethanechol, which has been reported to selectively stimulate  $M_3$  receptor [45,46,49].

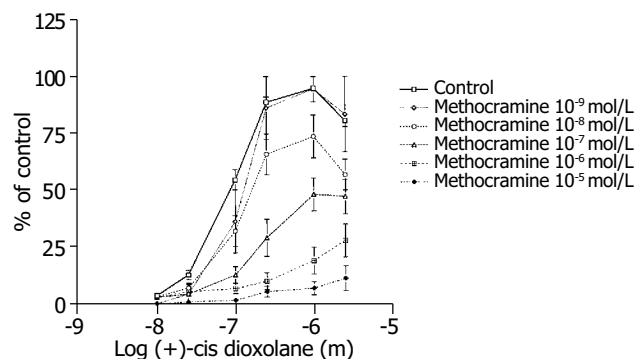
**Pirenzepine**, the  $M_1$  muscarinic receptor antagonist, was used at the concentrations from  $3 \times 10^{-5}$  to  $10^{-8}$  mol/L. Pirenzepine caused a significant inhibition of the of the contractile muscle response to the (+)-cis-dioxolane (Figure 5).



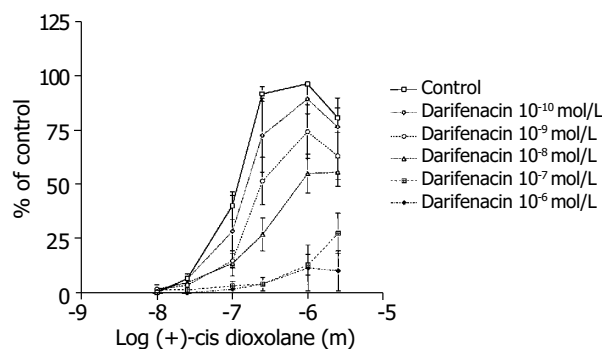
**Figure 5** Concentration-response curves for the contractile effect of (+)-cis-Dioxolane alone and in combination with different concentrations of  $M_1$  antagonist, pirenzepine ( $10^{-8}$  mol/L to  $10^{-5}$  mol/L) on the rat antral circular smooth muscle strips. Each point represents the mean  $\pm$  SE,  $n = 6, 6, 7$  and 8 strips. Cumulative concentration-effect curve was constructed for each strip for (+)-cis-Dioxolane, ( $10^{-8}$  to  $3 \times 10^{-5}$  mol/L). After washing, strips were equilibrated in either the absence (control) or presence of pirenzepine for 90 min. Subsequently, the second concentration-effect curve for (+)-cis-Dioxolane was constructed for each strip. Pirenzepine caused a significant inhibition of the contractile muscle response to (+)-cis-dioxolane.

Methocramine- $M_2$  muscarinic receptor antagonist was used at the concentration range between  $3 \times 10^{-5}$  and  $10^{-8}$  mol/L. Methocramine caused a significant inhibition of the of the contractile muscle response to the (+)-cis-dioxolane (Figure 6).

Darifenacin was used at the concentration range from  $3 \times 10^{-5}$  to  $10^{-9}$  mol/L (Figure 7). Darifenacin caused a significant inhibition of the contractile muscle response to the (+)-cis-dioxolane.



**Figure 6** Concentration-response curves for the contractile effect of (+)-cis-Dioxolane alone and in combination with different concentrations of  $M_2$  antagonist, methocramine (from  $10^{-8}$  mol/L to  $10^{-5}$  mol/L) on the rat antral circular smooth muscle strips. Each point represents the mean  $\pm$  SE;  $n = 4, 9, 10$  and 11 strips. First, cumulative concentration-effect curve was constructed for each strip for (+)-cis-Dioxolane, ( $10^{-8}$  to  $3 \times 10^{-5}$  mol/L). After washing, tissues were equilibrated in either the absence (control) or presence of methocramine for a 90 min. Subsequently, the second concentration-effect curve for (+)-cis-Dioxolane was constructed for each strip. Methocramine caused a significant inhibition of the contractile muscle response to (+)-cis-Dioxolane.

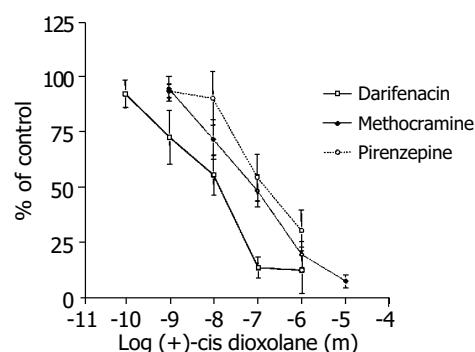


**Figure 7** Concentration-response curves for the contractile effect of (+)-cis-Dioxolane alone and in combination with different concentrations of  $M_3$  antagonist, darifenacin (from  $10^{-10}$  mol/L to  $10^{-6}$  mol/L) on the rat antral circular smooth muscle strips. Each point represents the mean  $\pm$  SE;  $n = 4, 7, 11, 10$  strips. First, cumulative concentration-effect curve was constructed for each strip for (+)-cis-Dioxolane, ( $10^{-8}$  to  $3 \times 10^{-5}$  mol/L). After wash, tissues were equilibrated in either the absence (control) or presence of darifenacin for a 90 min. Subsequently, the second concentration-effect curve for (+)-cis-Dioxolane was constructed for each strip. Darifenacin caused a significant inhibition of the contractile muscle response to (+)-cis-Dioxolane.

#### Comparison the muscarinic $M_1$ , $M_2$ and $M_3$ receptor antagonists mediating the rat antral circular smooth muscle contraction

All three receptors ( $M_1$ ,  $M_2$  and  $M_3$ ) subtype antagonists inhibited the antral circular muscle contraction to cholinergic agonist (+)-cis-dioxolane. Darifenacin ( $M_3$ ) was most potent in inhibiting rat antral smooth muscle cholinergic contraction. The dose-response curves were shifted to the right by muscarinic antagonists in the following order of affinity: darifenacin ( $M_3$ )

>methocramine ( $M_2$ ) >pirenzepine ( $M_1$ ). The  $EC_{50}$  for each antagonist was as follows: darifenacin 7.9, methocramine 7.2 and pirenzepine 6.8 (Figure 8).



**Figure 8** Comparison of the inhibition of the contractile response to cis-Dioxolane by 3 muscarinic receptor antagonists:  $M_3$ - darifenacin,  $M_2$ - methocramine, and  $M_1$ - pirenzepine. The  $M_3$  antagonist, darifenacin, was the most potent inhibitor of the contraction induced by (+)-cis Dioxolane ( $10^{-6}$  mol/L). The  $EC_{50}$  values for each were: for darifenacin ( $M_3$ ) -7.9; for methocramine ( $M_2$ ) -7.2; and for pirenzepine ( $M_1$ ) -6.8.

## DISCUSSION

It is well established that cholinergic agonists are of major importance for the stimulation of antral smooth muscle contraction and that their effect is mediated by muscarinic receptors. In various smooth muscle cells, the action of cholinergic agonists involved the occupation of specific receptors, activation of receptor-coupled G-proteins and subsequent activation of the second messenger systems, which would lead to the mobilization of  $Ca^{2+}$  from intracellular storage pools<sup>[10]</sup>. Certain steps of the signaling pathway (s) for the cholinergic contraction of antral circular smooth muscle in rats are still not understood, for example, the second messenger system or the functional characteristics of the muscarinic receptor subtypes involved. In our study the cholinergic rat antral circular muscle contraction *in vitro* was sensitive to the depletion from the extracellular  $Ca^{2+}$  that significantly diminished the cholinergic contraction. This finding along with the inhibitory effect of nifedipine indicated a dependence on extracellular  $Ca^{2+}$  influx via L-type calcium channels<sup>[50]</sup>.

It is still not clear whether cholinergic contractile pathways of antral circular smooth muscle are mediated by PTX-sensitive GTP-binding protein through an increased inflow of extracellular calcium, or mediated via PTX-insensitive, inositol triphosphate ( $IP_3$ )-dependent pathway. The circular muscle of cat esophagus and the lower esophageal sphincter (LES) were reported to be linked to different pathways. LES circular muscle cholinergic contraction was shown to be linked to the PTX-insensitive pathway, whereas esophageal circular muscle cholinergic contraction has been reported to be linked to the PTX-sensitive pathway<sup>[11-14]</sup>.

In the present study, pretreatment with PTX significantly inhibited the contractile response to bethanechol of the antral smooth muscle strips in the organ bath and of the dissociated myocytes.

More than one muscarinic receptor may be responsible for cholinergically mediated contractility at a particular area<sup>[6,22,51-56]</sup>. A major role for  $M_3$  and a minor role for  $M_2$  receptors in cholinergic-induced smooth muscle contraction have been shown at the tissue of various species and organs<sup>[57-59]</sup>, but it is still not clear how different receptors may interact to mediate a specific function. For example,  $M_2$  receptor might serve as an inhibitory presynaptic autoreceptor<sup>[60]</sup>, or  $M_2$  receptor could act by gating cation channels that were subsequently modulated by  $M_3$  receptors<sup>[61]</sup>.

The functional characteristics of muscarinic receptor subtypes regulating antrum circular muscle cholinergic contraction have not been previously reported. In our functional studies, cholinergic contraction in response to cis-dioxolane in rat antral circular muscles was mediated mostly through  $M_3$ , less by  $M_2$ , and the least by  $M_1$  muscarinic receptor subtypes.

The involvement of PTX-sensitive GTP-binding proteins in mediating the contractile response to muscarinic agonists has been reported to be linked to muscarinic  $M_2$  receptor in many studies, such as in cat esophageal circular muscle<sup>[11,12]</sup>. In contrast, our experiments in rat antral circular muscles indicated that the  $M_3$  muscarinic receptor was functionally prevalent, and yet, PTX-sensitive pathways were shown to be activated in the process of antral cholinergic contraction. A few published reports were in agreement with our results<sup>[13,22,26]</sup>.

Possible explanations of the reported differences include: (1) Different experimental systems were used to compare the homogenous suspension of smooth muscle cells was compared with smooth muscle strips. (2)  $M_3$  and  $M_2$  receptors were expressed together at the shared site of the signaling pathways<sup>[22,54,57,60,62]</sup>. (3) The cholinergic agonist (+/- cis-Dioxolane) was used in our studies. Although, it has been reported to be non-selective<sup>[46,48,52]</sup>, +/- cis-Dioxolane might be more selective for  $M_3$  muscarinic receptor than it was originally described<sup>[63]</sup>. (4) It has been suggested that seven transmembrane spanning receptors including muscarinic are promiscuous in that they could form interactions with multiple G-proteins<sup>[64]</sup> or activate many different transduction pathways at the same time<sup>[65]</sup>. (5)  $M_2$  receptor might be masked by  $M_3$  receptor, thus detectable when  $M_3$  receptor was inhibited. Separating out these possibilities would require more specific antagonists than the currently available.

In conclusion, the rat antral circular smooth muscle contracts to cholinergic agonists (bethanechol chloride and (+)-cis-Dioxolane) in a dose-dependent fashion through activation of muscarinic receptors at the smooth muscle. The contractile responses to cholinergic agonists are dependent on the increase in intracellular calcium induced by the influx of extracellular calcium via L-type voltage gated calcium channels. Bethanechol activates  $M_3$  and  $M_2$  muscarinic receptors coupled to pertussis-toxin sensitive GTP-binding protein (s). The presence of a residual contractile cholinergic response after 5 and 10 min in calcium-free buffer, as well as a residual contractile response after the treatment with nifedipine, suggests a role for an additional pathway (s).  $M_3$  receptor is predominant out of three functionally tested  $M_1$ ,  $M_2$ , and  $M_3$  muscarinic receptors regulating cholinergic contraction. The involvement of more than one muscarinic receptor indicates more than one pathway (s) regulating the cholinergic contraction of rat antrum circular smooth muscle.

The results of these studies have important clinical implications for possible treatment of gastric dysfunction with muscarinic subtype-selective agents.

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