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Muscarinic Receptor Subtype Controlling the Carbachol-Induced Muscle Contraction in Guinea Pig Gastric Antrum

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Stimulation of muscarinic receptors by carbachol (CCh) in the circular smooth muscle of the guinea pig gastric antrum causes muscle contraction. In the present study, muscarinic receptor subtype controlling the muscle contraction in response to CCh was studied using putative muscarinic receptor antagonists. Isometric force of the isolated circular muscle strips was measured in an organ bath. CCh contracted the muscle in a dose-dependent way, and each of the three muscarinic receptor antagonists, 4-diphenylacetoxy-N-methylpeperdine methiodide (4-DAMP), methoctramine and pirenzepine shifted the concentration-response curves to the right without significantly reducing the maximum force. The affinities of the muscarinic antagonists (pA_2 values) obtained from Schild plot analysis were 10.15, 7.05 and 6.84 for 4-DAMP, methoctramine and pirenzepine, respectively. These results suggest that the M_3-subtype mainly mediate the muscle contraction in response to CCh in guinea pig gastric antrum.

Key Words: Muscarinic receptor subtype, Carbachol, Smooth muscle

INTRODUCTION

In the gastrointestinal (GI) smooth muscles, acetylcholine is the major excitatory neurotransmitter mediating muscle contraction. The muscarinic receptors are members of the superfamily of GTP binding protein-coupled receptors, predicted to have seven transmembrane domains (Brann et al, 1993). At present, five muscarinic receptor genes (m1~m5) have been cloned and sequenced. At least four subtypes of the muscarinic receptors (M_1~M_4) have been identified pharmacologically, whereas the pharmacological distinguish of the m5 gene product from the other subtypes are not sufficient (Eglen et al, 1994; Felder, 1995; Ehlert et al, 1997). Of the five muscarinic receptor genes, M_1, M_3 and M_5 muscarinic receptors appear to couple predominantly to the stimulation of phosphoinositide hydrolysis *via* pertussis toxin (PTX)-insensitive G proteins of the $G_{q/11}$ family. Whereas the M_2 and M_4 receptors appear to couple predominantly to the inhibition of adenylate cyclase *via* PTX-sensitive G proteins of the $G_{i/o}$ family (Zhang & Buxton, 1991; Eglen et al, 1994; Burford et al, 1995; Reddy et al, 1995; Ehlert et al, 1997).

According to a functional and a radioligand binding studies, many GI smooth muscles exhibit a preponderance of the M_2-subtype (~80%) with a minor population of the M_3-subtype (~20%) (Giraldo et al, 1987; Michel & Whiting, 1988; Zhang et al, 1991). In guinea pig gastric antral smooth muscle, it has been well proved that the stimulation of muscarinic receptors by carbachol (CCh) opens nonselective cation channel that admit an inward current of mainly Na^+ under physiological condition (Kim et al, 1995a; Kim et al, 1998). More recently, we reported the muscarinic receptor subtype that controlling cationic channel opening (the M_2-subtype) in guinea pig gastric antrum by evaluating pharmacological affinity profiles of muscarinic agonists and antagonists (Rhee et al, 2000). Nonetheless, in guinea pig gastric antrum, the feature of the muscarinic receptor subtypes mediating muscle contraction in response to CCh is still obscure. In the present study, therefore, we aimed to determine the receptor subtype controlling muscle contraction in guinea pig gastric smooth muscle by evaluating pharmacological affinity profiles of the

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antagonists for muscarinic receptors.

METHODS

Measurement of contractile force in isolated muscle strips

Guinea pigs (150~200 g) of either sex were exsanguinated after stunning. The antral part of the stomach was cut, and mucosal layer was separated from the muscle layers in bicarbonated-buffered Tyrode's solution containing 116 mM NaCl, 5.4 mM KCl, 24 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.6 mM glucose (pH 7.35 with HCl). Muscle strips (2~3 mm wide, 10~12 mm long) from the antral part were cut parallel to the circular fibers and placed in a vertical organ chamber (50 ml) containing bicarbonate-buffered Tyrode solution, which was maintained at 36.5°C and gassed with 5% CO_2 in O_2. One end of the strip was mounted on platinum steel tissue hook and the other was connected to the force transducer (Harvard, U.K.) to measure isometric contraction. A 1 g load was placed on each preparation and the tissues were equilibrated for more than 60 min. A cumulative concentration-response curve to CCh was established. After attaining control responses, strips were washed several times with fresh Tyrode's solution and treated for more than 5 min with antagonists for muscarinic receptor (4-DAMP, methoctramine and pirenzepine), and dose-response curves for CCh were obtained again in the presence of the antagonists. Contractions were normalized to the maximal contractile forces in each tissue during the first exposure to CCh.

Data analysis

The data are presented as the mean±S.E.M. with n, the sample size. Statistical significance was estimated by Student's paired or unpaired t-test. P values of less than 0.05 were considered statistically significant. Log EC_{50} values were determined by fitting lines of the following logistic sigmoid function of the log concentration-response curves: $F/F_{max}=\{1+([EC_{50}]/[A])^h\}^{-1}$, where F is the force generated at a given CCh concentration, F_{max} is the force at a maximal CCh concentration. EC_{50} is the agonist concentration ([A]) when F was 50% of F_{max} and h is the slope factor of the agonist curve. Schild analysis (Arunlakshana & Schild, 1959) was performed by calculating dose ratios (DR) at equieffective concentrations of CCh (EC_{50} concentration) for each concentration of antagonist, and plotting log (DR-1) *vs* -log [B], where $DR=[A_2]/[A_1]$, $[A_1]$ is the EC_{50} concentration of CCh in the absence of antagonist, $[A_2]$ is the EC_{50} concentration of CCh in the presence of antagonist and [B] is the concentration of antagonist. As a consequence of the variable number of experiments performed with the various concentrations of antagonist, the DR for each antagonist concentration was derived from the mean concentration-response curves that incorporated all data for each experimental group. This resulted in a single value without S.E.M. for the log (DR-1) at each antagonist concentration in the Schild plots. Linear regression analysis of the Schild plots was performed, which allowed estimation of the slope and intercepts. The pA_2 value, defined as the negative logarithm of the antagonist concentration that produces a 2-fold rightward shift in the concentration-response curve, was extrapolated from the regression equation as the χ-intercept.

Chemicals

4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), pirenzepine hydrochloride and methoctramine hydrochloride were obtained from RBI (Natick, MA, U.S.A.); carbamylcholine chloride (carbachol; CCh) and all other chemicals were from Sigma (St. Louis, MO, U.S.A.).

RESULTS

Muscarinic receptor subtype mediating CCh-induced muscle contraction

Muscle strips were stimulated with ascending concentration of CCh in a cumulative fashion (Fig. 1). CCh increased the contractile force in a dose-dependent way, and the maximum contractile force was obtained at around 0.5 μM. In response to CCh above 0.5 μM, amplitudes of the phasic contraction were not maintained, but decreased gradually. After the attainment of control response to CCh, the antagonism by 4-DAMP, methoctramine and pirenzepine were observed. Fig. 1 shows the examples of the effects of antagonists on the muscle contraction in response to CCh. All the antagonists shifted the

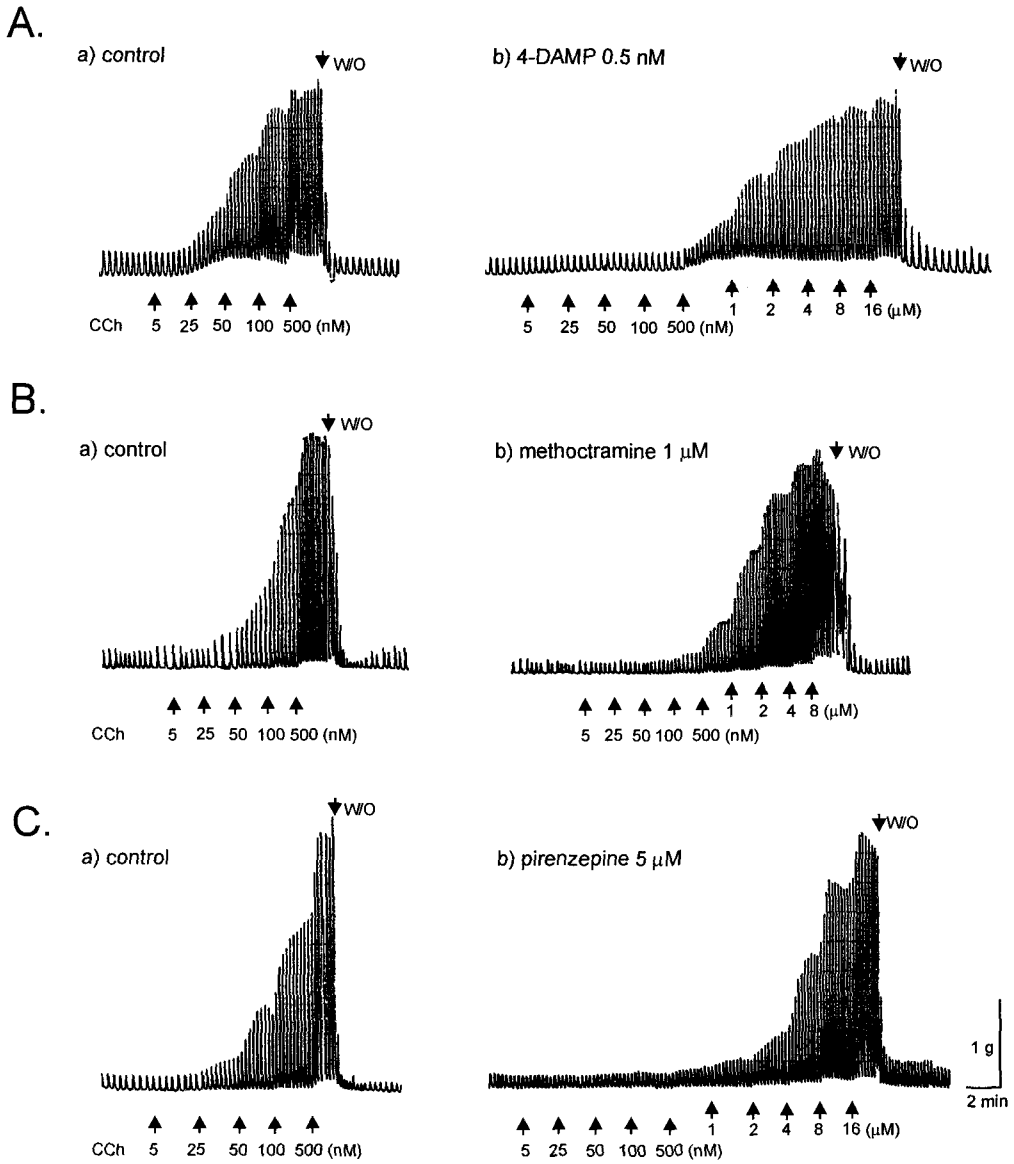

Fig. 1. Original traces show the effects of muscarinic antagonists for the contraction in response to carbachol (CCh). Series of ascending concentrations of CCh was applied onto the muscles. 4-DAMP (A), methoctramine (B) and pirenzepine (C) competitively inhibited the CCh responses without significantly reducing the maximal contraction. Concentrations of the antagonists were depicted in the figures.

concentration-response curve to the right without significantly reducing the maximum responses. The EC_{50} changed from 65 ± 2.8 nM to 0.36 ± 0.03, 1.12 ± 0.07, 2.82 ± 0.13 and 20.0 ± 1.3 μM in the presence of 0.25, 0.5, 1.0 and 5 nM 4-DAMP, respectively (Fig. 2A, n=14). The calculated EC_{50} (94 ± 4.2 nM) for CCh was shifted to 0.20 ± 0.07, 1.12 ± 0.12 and 3.98 ± 0.14 μM at 0.1, 1.0 and 5.0 μM methoctramine, respectively (Fig. 3A, n=14). EC_{50} shifted from 50.8 ± 1.3 nM to 0.28 ± 0.04, 1.8 ± 0.09 and 30 ± 2.2 μM in 0.5, 5 and 40 μM pirenzepine, respectively (Fig. 4A, n=8). When a Schild plot was constructed, the slope was not significantly different from unity and when constrained to unity gave pA_2 values of 10.15, 7.05 and 6.84 for 4-DAMP, methoctramine and pirenzepine, respectively (Figs. 2B, 3B, 4B). The pA_2 values obtained from each antagonist were summarized and compared with those described in other preparations (Table 1).

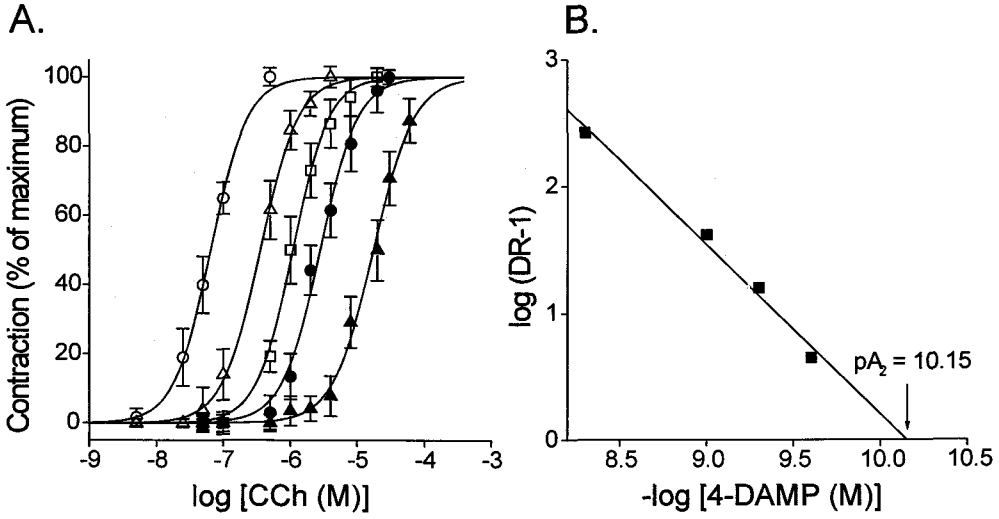

Fig. 2. Inhibition of carbachol-induced contraction in circular muscle of guinea pig gastric antrum by 4-DAMP. (A) Concentration-response curve of the control (○) was shifted to the right in a parallel manner by 0.25 (△), 0.5 (□), 1.0 (●) and 5 nM (▲) 4-DAMP (n=14). The slopes of each curves were 1.58 (○), 1.55 (△), 1.62 (□), 1.55 (●) and 1.45 (▲), and they were statistically insignificant among groups. (B) Schild plot depicting antagonism of CCh-induced muscle contraction by 4-DAMP; apparent pA_2=10.15 (slope= −1.33, r^2=1.0).

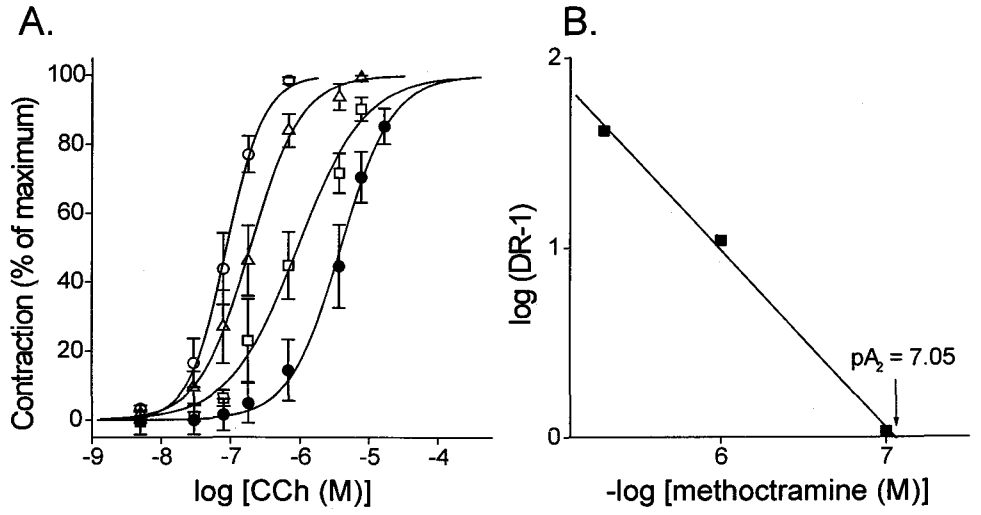

Fig. 3. Inhibition of carbachol-induced contraction by methoctramine. (A) Concentration-response curve of the control (○) was shifted to the right in a parallel manner by 0.1 (△), 1 (□) and 5 μM (●) methoctramine (n=14). The slopes of each curves were 1.53 (○), 1.20 (△), 1.0 (□) and 1.20 (●), and they were statistically insignificant among groups. (B) Schild plot depicting antagonism of CCh-induced muscle contraction by methoctramine; apparent pA_2=7.05 (slope= −0.94, r^2=1.0).

DISCUSSION

Muscarinic receptors exist in multiple subtypes and many tissues express more than one subtype, which may couple to different intracellular effectors and thus have different physiological roles (Zhang & Buxton, 1991; Eglen et al, 1994; Thomas & Ehlert, 1994; Burford et al, 1995; Felder, 1995; Ehlert et al, 1997).

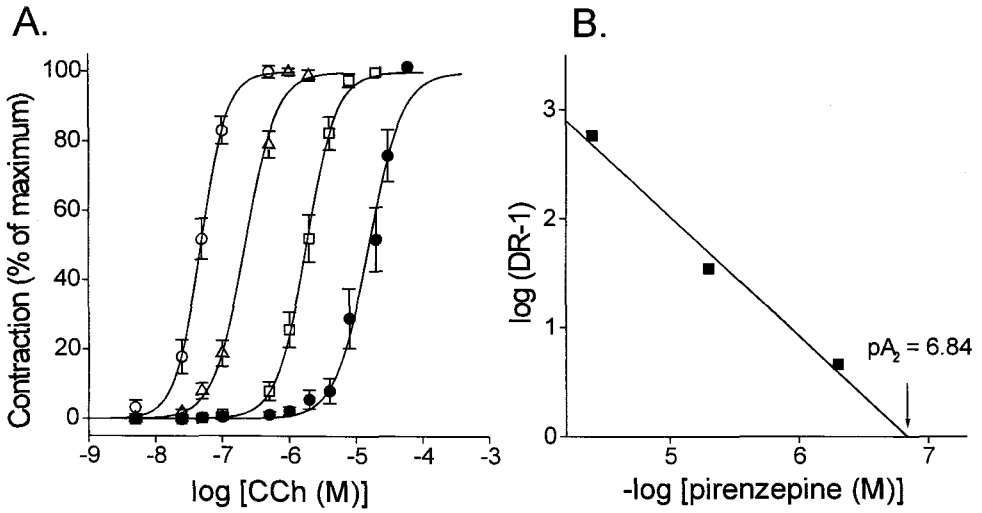

Fig. 4. Inhibition of carbachol-induced contraction by pirenzepine. (A) concentration-response curve of the control (○) was shifted to the right in a parallel manner by 0.5 (△), 5 (□) and 40 μM (●) pirenzepine (n=8). The slopes of each curves were 2.1 (○), 1.85 (△), 1.94 (□) and 1.72 (●), and they were statistically insignificant among groups. (B) Schild plot depicting antagonism of CCh-induced muscle contraction by pirenzepine; apparent pA_2=6.84 (slope=−1.10, r^2=1.0).

Table 1. Comparison of the affinities of the muscarinic antagonists (pA_2 values) obtained at receptors mediating CCh-induced muscle contraction in guinea-pig gastric antrum with those selected values from Eglen et al (1994)

Compound	Present study	Subtypes			
		M_1	M_2	M_3	M_4
Pirenzepine	6.84	8.1~8.5	6.7	6.7~7.1	7.7~8.1
Methoctramine	7.05	7.1~7.6	7.8~8.3	6.3~6.9	7.6
4-DAMP	10.15	8.9~9.2	8.0~8.4	8.9~9.3	9.4

To date, progress in the identification of selective agonists or antagonists for each muscarinic receptor subtype has been largely unsuccessful. This limited selectivity of muscarinic ligands renders difficulty in characterization of each subtype. In spite of these difficulties, the available ligands can be exploited to allow pharmacological characterization of muscarinic receptor populations. This is most frequently achieved by generating rank order of affinities for a series of compounds (Dorje et al, 1991; Zhang et al, 1991). Based on this strategy, we used putative antagonists to determine the subtypes of muscarinic receptor mediating CCh-activated smooth muscle contraction from the guinea pig stomach.

The CCh-induced contraction observed in the present experiments is inhibited by the muscarinic antagonists with the affinity sequence: 4-DAMP (10.15) >> methoctramine (7.05) ≥ pirenzepine (6.84). This rank order of antagonist affinity is consistent with the activation of the M_3-subtype (Eglen et al, 1994). A putative M_1-selective antagonist, pirenzepine had the pA_2 value of 6.84, which is closer to the reported values for the M_2 (~6.7) or the M_3 subtype (6.7~7.1) rather than either the M_1 (8.1~8.5) or the M_4 (7.7~8.1) subtype (Table 1). This indicates that the M_1 and/or the M_4 subtypes might not be involved in the CCh-induced contraction in our preparation. Furthermore, obtained pA_2 value of methoctramine

(7.05), a putative M_2-selective antagonist, is similar to the M_3-mediated response in other preparation (6.3~6.9). A putative M_3-selective antagonist, 4-DAMP showed high affinity (pA_2=10.15), which is approximately ten times higher than that of previously reported values (8.9~9.3) obtained from the functional or binding studies in other cells (Eglen et al, 1994). Although this discrepancy of the affinity on muscarinic receptors in guinea pig stomach remains to be elucidated, the strong action of 4-DAMP on the receptor as well as the affinity sequence suggests that the contractile effects of CCh on the muscle were predominantly mediated by the M_3-subtype.

Parekh & Brading (1992) suggested that the M_3-subtype mediate CCh-induced contraction in circular muscle of guinea-pig gastric fundus. Therefore, it is strongly suggested that the same muscarinic receptor subtype (M_3) predominantly mediate the CCh-induced muscle contraction in the whole body of the guinea pig stomach. The M_3-subtype-mediated muscle contraction has been reported from the variety of visceral and vascular smooth muscles (Ehlert et al, 1997 and references therein). Therefore, we suggest that the M_3-subtype is the major contributing factor for contracting visceral smooth muscle including gastric muscles.

Although the predominant role of the M_3-subtype has been suggested in the present experiments, the contribution of the M_2-subtype in CCh-mediated muscle contraction should not be excluded because the stimulation of the M_2-subtype by CCh depolarizes membrane potential *via* opening of the nonselective cation channels (Rhee et al, 2000). Membrane depolarization by CCh increases the amplitudes of electrical slow waves and thus Ca^{2+} influx through the voltage-dependent Ca^{2+} channels (Kim et al, 1995). Furthermore, in guinea pig gastric smooth muscle cells, it has been demonstrated that Ca^{2+} influx through the CCh-activated nonselective cation channels induces a rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) under physiological condition (Kim et al, 1998). Therefore, if CCh stimulates the M_2 and the M_3 receptors at the same time, it follows that the two contractions should be additive. In the present experiments, a putative M_2 antagonist, methoctramine antagonized the contractile responses to CCh with pA_2 value of 7.05 that was intermediate between those expected for the individual M_2 (7.8~8.3) and M_3 (6.3~6.9) subtypes (Eglen et al, 1994), but closer to the M_3 receptor. These results may indicate that the M_2-subtype partly contributes to the overall response in guinea pig gastric smooth muscle, but do not play a predominant role. This situation might be expected if CCh stimulates both the M_2 and the M_3 receptors at the same time. In spite of this possibility, we suppose that the role of the M_2-subtype in the CCh-induced muscle contraction is somewhat weak because of the following reason. It has been suggested that different muscarinic receptors may provide different contributions to the contraction depending on the agonist concentration (Zholos & Bolton, 1997). In the present study, CCh produced contraction of the guinea pig gastric antrum with EC_{50} values in the nanomolar range (50~100 nM) and it is this part that is a subject of functional pharmacological studies. It is possible that the M_3-subtype/PLC/IP_3 system is relatively more important at these concentrations because membrane depolarization or cationic current in this tissue requires somewhat higher agonist concentration. A steep increase in the cationic current activated by the M_2-subtype was seen over the range 0.5 to 10 μM CCh (Rhee et al, 2000). It seems that, therefore, in the concentration range of CCh used in the present study (5 nM~0.5 μM), the roles of M_2-mediated cationic current and the corresponding membrane depolarization are insignificant.

In summary, our results show that muscarinic receptors mediating muscle contraction in guinea pig gastric antrum have different affinities to the various antagonists for muscarinic receptors. The receptor mediating muscle contraction fits the description of the M_3-subtype.

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