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Research Article

Pharmacological Characterization of Muscarinic Receptor Subtypes Involved in Carbachol-Induced Contraction of the Chicken Proventriculus

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Abstract

Present study was designed to characterize the postjunctional muscarinic acetylcholine receptor mediating contraction of the chicken proventriculus by using muscarinic receptor agonists and antagonists. Muscarinic agonists caused concentration-dependent contraction. The ranking order of pEC_{50} values were muscarine > arecaidine propargyl ester > oxotremorine M > carbachol > methacholine > arecoline > acetylcholine > bethanechol = McN-A343 = pilocarpine. The responses to carbachol were competitively antagonized by AF-DX116 (pK_b=5.9), AF-DX384 (6.84), 4-DAMP (8.79), methoctramine (5.81), pirenzepine (6.78), p-F-HHSID (7.41), atropine (8.43),tropicamide (6.91), himbacine (7.01), AQ-RA741 (6.6) and dimethindene (6.73). The correlation of pK_b of the chicken proventriculus with that of the mammalian M_3 receptor was the highest among five muscarinic receptors. The proventriculus membrane contained a single class of [^{3}H]-quinuclidinyl benzilate binding sites. pK_i values of the antagonists in a binding displacement study correlated with that of the M_3 subtype. Pertussis toxin(100μg/kg, i.p. for 72h) significantly decreased the contraction induced by a low concentration of carbachol. In 4-DAMP mustard and AF-DX116-pretreated muscle strips, AF-DX116 shifted the concentration-response curve for oxotremorine M to the right in the presence of 5-hydroxytryptamine and forskolin. The pK_b value (6.22) was significantly higher than that obtained in the normal condition (5.78). Isoproterenol increased cyclic AMP, and carbachol significantly decreased the isoproterenol-induced increase in cyclic AMP. The results indicated that a muscarinic agonist mainly acts on the M_3 receptor to cause contraction of the chicken proventriculus but the possible involvement of M_2/M_4 receptors cannot be excluded as demonstrated in mammalian gastrointestinal tract.

Introduction

Muscarinic acetylcholine receptors belong to a family of G protein-coupled receptors. Molecular cloning studies have demonstrated the existence of five subtypes, termed m_1, m_2, m_3, m_4 and m_5 which correspond to functional M_1, M_2, M_3, M_4 and M_5 receptors. Studies of the expression of cloned receptors in various cell lines have shown that individual subtypes preferentially interact with specific signal transduction pathways. M_1, M_3 and M_5 couple with $G_{q/11}$ protein and stimulate phosphoinositide turnover produced IP_3 and diacylglycerol, while M_2 and M_4 couple with Gi protein, resulting in a decrease of adenylate cyclase activity [1-5].

Functional studies using electrical field stimulation and extrinsic nerve stimulation have provided evidence for an important role of parasympathetic cholinergic neurons and postsynaptic muscarinic receptors in gastrointestinal smooth muscle contraction. Of the five muscarinic receptor subtypes, both M_2 and M_3 are abundantly expressed in smooth muscle throughout the gastrointestinal tract [3,5,6]. Radioligand binding [7], mRNA hybridization [8] and immunoprecipitation studies [9] have shown that the M_2 receptor is more abundant of the two receptors in gastrointestinal smooth muscle (M_2:M_3= from 3:1 to 5:1). However, despite the predominant expression of the M_2 receptor, pharmacological analysis of carbachol-induced contraction with muscarinic receptor antagonists has shown the involvement of the M_3 receptor in contractile responses in the gastrointestinal tract [3,6,10-12]. Since an increase in cytoplasmic cyclic AMP causes relaxation of smooth muscle, the M_2 receptor-mediated decrease in cyclic AMP synthesis enhances contraction of the gastrointestinal tract by M_3 receptor activation. Therefore, M_2 receptor activation is thought to elicit its contractile action indirectly [6,13,14]. However, in M_3 receptor knockout mice, M_2 receptor activation directly caused gastrointestinal contraction [15,16] and played an essential role in the generations of rhythmic motor activity [17].

Muscarinic receptor agonists also caused contraction of the gastrointestinal tract of lower non-mammalian vertebrates such as chickens, bullfrogs and goldfish, and the non-selective muscarinic receptor antagonist atropine decreased contractile responses to acetylcholine or carbachol, indicating the involvement of muscarinic receptors [18-20]. Nouchi et al. [21] have already demonstrated M_4-mediated negative and M_1-mediated positive inotropic responses in

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the chicken heart, which were different from inotropic responses observed in the mouse atrium (negative inotropic responses were mediated by M_2 receptor and positive inotropic responses were mediated by M_3 type) [22]. These findings suggested phylogenic differences in the expression of muscarinic receptor subtypes in the gastrointestinal tract of non-mammalian vertebrates such as chicken. Characteristics of gastrointestinal muscarinic receptor subtypes have been already examined in the ileum of chicken and M_3 receptor mediated contraction has been demonstrated [23]. Although in the mouse gastrointestinal tracts, contribution of M_2 and M_3 receptors in the muscarinic contraction in the stomach and small intestine was almost the same [15,16], it was not clear that similarity of muscarinic receptor subtypes involved in the contraction of stomach and intestine is extrapolated to another vertebrates such as chicken.

The aim of the present study was to clarify the muscarinic receptor subtypes involved in carbachol-induced contraction of the chicken proventriculus (stomach) and to compare the results with those in the ileum [23]. Muscarinic receptor agonists and antagonists were used in the contraction study, and [^{3}H]-quinuclidinyl benzilate (QNB) binding in the proventriculus smooth muscle membrane was analyzed. To examine the presence of even number of muscarinic receptors (M_2/M_4), the effect of carbachol on isoproterenol (β–adrenoceptor against)-induced increase in cytoplasmic cyclic AMP was examined.

Materials and Methods

Animals and tissue preparations

All experiments were performed in accordance with institutional guidelines for animal care at Rakuno Gakuen University. Male white Leghorn chickens (Gallus domesticus), aged 2-5 weeks (80-120 g), were anesthetized with isoflurane, stunned, and bled to death. The proventriculus (located adjacent to the lower esophagus and corresponding to the gastric fundus in mammals) was removed after a midline incision, and the luminal contents were flushed out using cold Krebs solution. The proventriculus was cut open along the longitudinal axis. After removal of the mucosa, longitudinal muscle strips were prepared by surgically cutting through the muscle coat parallel to the direction of longitudinal muscle fibers.

Contraction study

The proventriculus strips (1 x 15 mm, 10-20 mg) were suspended vertically in an organ bath (5ml) to measure longitudinal muscle contraction. The organ bath contained warmed (37^{0}C) Krebs solution (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.5 equilibrated with 95% O_2 + 5% CO_2 (pH 7.4). Mechanical activity was measured with an isometric force transducer (TB-612T, Nihon Kohden, Tokyo, Japan) and recorded on an ink-writing recorder. Initial load was set at 0.5g. The preparations were rinsed with Krebs solution every 15 min and allowed to equilibrate for 1h. Prior to the addition of muscarinic receptor agonists, the preparations were subjected to 3 or 4 stimulations with 50 mM KCl until a reproducible contraction was obtained. To compare contractile responses to muscarinic receptor agonists, cumulative concentration-response curves were established with half log-unit ascending concentration increments (1nM-10μM). The EC_{50} values and amplitudes of maximum contraction were calculated from computer fits based on non-linear regression, and they were used as parameters for comparison of agonist affinity. The amplitude of contractions among the preparations was normalized by a standard contraction of 50 mM KCl and expressed as percentage.

The effects of muscarinic receptor antagonists on carbachol-induced contraction were examined to characterize muscarinic receptor subtypes. The pK_b value was determined for each antagonist according to the procedure of Arunlakshana and Schild [24]. Briefly, concentration-response curves for carbachol were constructed in the absence and presence of three increasing concentrations of antagonists (30-min treatment). The concentration ratio (CR) of EC_{50} values was determined for each antagonist. If blockade occurs in a competitive manner, a plot of the logarithm of CR-1 against the logarithm of the molar concentration of the antagonist should yield a straight line, the slope of which is not different from unity (1.00), and the intercept on the abscissa will give pK_b.

In order to determine whether the pertussis toxin (PTX)-sensitive G-protein pathway is involved in muscarinic contraction, chickens were injected with 100μg/kg of PTX (dissolved in saline) intraperitoneally 72h before the study as previously described [15,25]. Proventriculus muscle strips from PTX-treated and control chickens in the tissue bath were exposed to carbachol, and the concentration-response curves were compared.

Possible involvement of the M_2 muscarinic receptor in the contractile response to an muscarinic receptor agonist (oxotremorine M) was investigated using proventriculus muscle strips pretreated with 4-DAMP mustard (100nM) and AF-DX116 (3 μM). 4-DAMP mustard caused irreversible alkylation of non-M_2 muscarinic receptors because the simultaneous presence of the selective M_2 receptor antagonist AF-DX116 protects the M_2 receptors from alkylation (for 1h). Following prolonged washout every 10min (90min), the muscle preparations were pre-contracted with 5-hydroxytryptamine (5μM) and then relaxed by forskolin (5μM) to resting muscle tension by activation of adenylate cyclase This method of receptor alkylation, followed by the addition of a spasmogenic agent and a relaxing agent, has already been used in several other mammalian tissues to investigate M_2 receptor functions [13,14,26]. In this experimental condition, concentration-response curves for oxotremorine M were established in the absence and presence of AF-DX116, and the pK_b value for AF-DX116 was compared with the control value.

Binding study

To characterize the muscarinic receptor in the chicken proventriculus, we carried out a receptor binding assay using [^{3}H]-QNB (42.0 Ci/mmol, PerkinElmer Life Science, USA). Isolated longitudinal muscle preparations of the proventriculus were cut into small pieces and homogenized in 10 volumes of ice-cold Tris-EDTA buffer solution (mM: Tris, 50; Na_2EDTA, 0.5; $MgSO_4$, neutralized with HCl to pH 7.4 at 4^{0}C) with the use of a Polytron. The homogenate was filtered through a single layer of nylon mesh (pore size, 250 µm) and centrifuged at 2000 g for 20 min at 4^{0}C, and the pellet was discarded. The supernatant was centrifuged at 40,000 g for 60 min at 4^{0}C. The resulting pellets were washed twice and suspended in the Tris-EDTA buffer and used as a crude membrane preparation for determination of [^{3}H]-QNB binding. Protein in the membrane preparation was measured according to the method of Lowry et al. [27]. The membrane preparation (200 µg protein per tube) was incubated with seven increasing concentrations (0.03-6.2 nM) of [^{3}H]-QNB in 500 µl

of Tris-EDTA buffer (at 37^{0}C for 60 min). The binding reaction was stopped by adding ice-cold Tris-EDTA buffer (4 ml), and the mixture was then filtered through a 0.3% polyethylenimine-presoaked glass fiber filter (GF/B; Whatman) under a vacuum to trap the crude membrane. The filter was then rapidly washed four times with 3 ml of ice-cold incubation buffer and placed in 20-ml glass scintillation vials with scintillation fluid (Scintisol EX-H, Dojin). Radioactivity trapped on the filter paper was measured by a liquid scintillation spectrometer (LCS-700; Aloka). Specific binding was calculated by subtracting non-specific binding from total binding. Non-specific binding was determined in the presence of 100 μM atropine. The maximum number of binding sites mg/protein (Bmax, concentration of receptors) and the equilibrium dissociation binding constant (K_d) were estimated by Scatchard analysis. Lines of the best fit were calculated using linear regression by the method of least squares. To characterize the [^{3}H]-QNB binding site (muscarinic receptor) in the chicken proventriculus, a displacement study was carried out using several muscarinic receptor antagonists. [^{3}H]-QNB (0.2-0.3 nM) and the crude membrane (200 μg protein) were incubated with various concentrations of muscarinic receptor antagonists (tropicamide, p-F-HHSID, pirenzepine, metochtramine, AF-DX 116, AF-DX 384, 4-DAMP, himbacine, atropine, AQ-RA 741 and dimethindene) for 60 min at 37^{0}C. After incubation, [^{3}H]-QNB bound on membrane muscarinic receptors was separated by filtration through 0.3% polyethylenimine-presoaked glass fiber filter and the radioactivity on the filters was measured. The displacement curves were analyzed using Graph Pad Prism (Ver. 4 Graph Pad Software, Inc.), and it was clarified whether one or two-site binding model fits the obtained data. From the IC_{50} value (concentration of an antagonist that inhibits 50% of the control binding obtained in the absence of displacers), the inhibition constant (K_i) was calculated by the equation of Cheng and Prusoff [28]: $K_i = IC_{50}/(1+[L]/K_d)$, where [L] is the concentration of [^{3}H]-QNB used in the displacement study. pK_i values for antagonists were compared with the reference pK_b/pK_i values of respective muscarinic receptor subtypes indicated in Table 1.

Table 1: Pharmacological characterization of the muscarinic receptor involved in the carbachol-induced contraction of the chicken proventriculus.

Antagonists	pKb	M1	M2	M3	M4	M5
Atropine	8.43	9	8.7	9.2	8.9	8.9
4-DAMP	8.79	9.2	8.1	8.7	9	9
AF-DX 116	5.9	6.6	7.1	5.9	6.8	6.5
AF-DX 384	6.84	7.3	9	7.2	8.7	6.3
Pirenzepine	6.78	8	6.3	6.8	7.1	6.9
Tropicamide	6.91	7.2	7.3	7.4	7.8	
AQ-RA 741	6.6	7.4	8.2	6.8	8.1	6.1
Himbacine	7.01	6.7	8	6.9	7.8	6.1
Metochtramine	5.81	6.7	7.7	6.1	7	6.3
p-F-HHSID	7.41	7.3	6.6	7.7	7.2	6.7
Dimethindene	6.73	7.08	7.78	6.7	7	

pK_b values for the muscarinic antagonists were obtained with inhibition of carbachol-induced contraction in the proventriculus. Reference antagonist affinity (pK_b/pK_i) of each antagonist is adopted from following papers: Bonner et al. [38], Gardner et al. [39], Buckley et al. [40], Giraldo et al. [41], Eglen and Whiting [42], Waelbroeck et al. [43], Dorje et al., [44], Dorje et al., [45], Liebmann et al. [46], Lazareno and Birdsall [47], Doods et al. [48], Eglen et al. [49], Pfaff et al. [50], Melchiorre et al. [51], Esqueda et al. [52], Shi et al. [53], Darroch et al. [23], Eglen and Nahorski [54], Nasman et al. [55], Wang et al. [56] and Lazareno et al. [57].

Cyclic AMP measurements

Measurements of cyclic AMP were made basically according to the method of Kitazawa et al. [29]. In brief, isolated longitudinal muscle strips of the proventriculus weighing approximately 5-10 mg were prepared and suspended in warmed (37^{0}C) and gassed (95% O_2-5% CO_2) Krebs solution containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (100 μM). To investigate the effect of carbachol on isoproterenol-induced increases in cyclic AMP levels, the muscle strips were incubated for 10 min in the presence or absence of carbachol. After this incubation, isoproterenol (1 μM) was added and allowed to act for 5min, and then the muscle strips were quickly frozen in liquid nitrogen and homogenized in 6% trichloroacetic acid solution with a Polytron. The homogenate was centrifuged at 2000g for 20min (twice) and the resulting supernatant was collected. The pellet was dissolved in 0.1 n NaOH for protein determination by the method of Lowry et al. [27]. After removing trichloroacetic acid in the supernatant by washing three times with water-saturated ether, cyclic AMP in the extract was assayed using an enzyme immunoassay kit (Amersham International Ltd, Piscataway, NJ, USA). Tissue cyclic AMP levels were expressed as picomoles per milligram of protein.

Chemicals

The following chemicals were used: (2-acetoxypropyl) trimethlammonium chloride (methacholine, Sigma), acetylcholine chloride (ACh,Wako), arecaidine propargylester hydrobromide (APE, Sigma), atropine sulfate (Sigma), carbamoylcholine chloride (carbachol, Sigma), carbamyl-β-methycholine chloride (bethanechol, Sigma), 4-[[[(3-Chlorophenyl)amino]carbonyl]oxy]-N,N,N-trimethyl-2-butyn-1-aminium chloride (McN-A343, Tocris), 11-[[4-[4-(diethylamino)butyl]-1-piperidinyl]acetyl]-5, 11-dihydro-6H-pyrido [2,3-b][1,4] benzodiazepine-6-one(AQ-RA741,Tocris), 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5, 11-dihydro-6H-pyrido [2,3b][1,4] benzodiazepine-6-one (AF-DX116,Tocris), N-[2-[2-[(dipropylamino)methyl]-1-peperidenyl]ethyl]-5, 6-dihydro-6-oxo-11H-pyrido[2,3-b][1,4]benzodiazepine-11-carboxamide (AF-DX384,Tocris), N, N-dimethyl-3-[(1S)-1-(2-pyridinyl)ethyl]-1H-indene-2-ethanamine meleate((S)-(+)-dimethindene maleate, Tocris), (3S,3aR,4R,4aS,8aR,9aS)-4-[{1E}-2-[(2R,6S)-1, 6-dimethyl-2-piperdinyl]ethenyl] decahydro-3-methyl-naphthol [2,3-c] furan-1 (3H)-one (himbacine, Sigma), 4-diphenylactoxy-N-methylpiperidine methiodide (4-DAMP, Sigma), (3S,4R)-4,5-dihydro-3-ethyl-4-(1-methyl-1H-imidazol-5-ylmethyl)-2(3H)-furanone hydrochloride (pilocarpine, Sigma), forskolin (Tocris), isoproterenol hydrochloride (Sigma), 5-hydroxytryptamine sulfate complex (Sigma), 1-methyl-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid methyl ester hydrobromide (arecoline, Sigma), (+)-(2S,4R,5S)-tetrahydro-4-hydroxy-N,N,N,5-tetramethyl-2-furanmethanammonium chloride (muscarine, Sigma), oxotremorine methiodide (oxotremorine M, Sigma), para-fluoro-hexahydrosila-difenidol (p-F-HHSID, Tocris), N-Etyl-2-phenyl-N-[4-pyridyl-methyl] hydrocrylamide (tropicamide, Sigma), pirenzepine dihydrochloride monohydrate (pirenzepine, Tocris) and methoctramine tetra-hydrochloride (methoctramine, Sigma). AF-DX 116, AF-DX 384 and AQ-RA 741 were dissolved in dimethyl sulfoxide, and himbacin was dissolved in ethanol. Other drugs were dissolved in distilled water. The administration volume of each drug was less than 1% of the bath volume except for KCl (2.5 % of the bath volume).

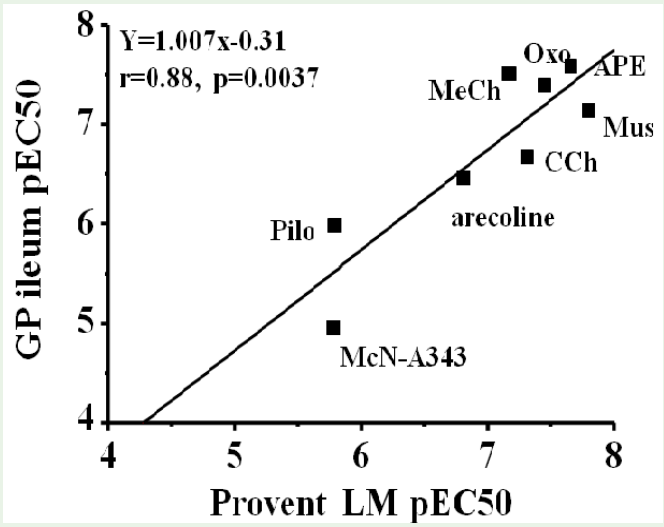

Figure 1: Correlation of pEC_{50} values of muscarinic agonists in the chicken proventriculus and guinea-pig ileum. pEC_{50} values of Arecaidine Propargyl Ester (APE), Methacholine (MeCh), Carbachol (CCh), Muscarine (Mus), Oxotremorine M (Oxo), McN-A343, Pilocarpine (Pilo) and arecoline obtained from the contraction study in the chicken proventriculus were plotted against those obtained in the guine-pig ileum [30], where the M_3 receptor mediates the contractile response. A significant correlation was found between pEC_{50} values obtained from the two smooth muscle preparations. Values of the chicken proventriculus are means of more than 4 experiments.

Statistical analysis

The results of experiments are expressed as means ±S.E.M of more than four experiments using muscle strips from different animals. The significance of differences between the values was determined at $P<0.05$ using Student's t-test (paired and unpaired) for single comparisons. Correlation between obtained pEC_{50}/pK_b and the documented pEC_{50}/pK_i values was analyzed, and the correlation coefficient, its probability and slope of the regression line were calculated using ORIGIN (Ver. 8.6.Origin Lab. MA.USA).

Results

Contractile responses to muscarinic receptor agonists

Cumulative application of carbachol (1 nM - 3μM) caused sustained contractions of isolated chicken proventriculus strips (pEC_{50}=7.29±0.02, maximum contraction=112±2.6%, n=17). The concentration-response relationships for carbachol did not change at 40 min interval construction of concentration-response curves (5 times). The contractile responses to carbachol were not affected by pretreatment with tetrodotoxin (1μM, pEC_{50}=7.24±0.06, maximum contraction=110±3.6%, n=7) or hexamathonium (100μM, pEC_{50}=7.22±0.03, maximum contraction=115±6.5%, n=5). Acetylcholine, arecoline, arecaidine propargyl ester (APE), bethanechol, oxotremorine M, pilocarpine, McN-A343, muscarine and methacholine also caused contraction of the proventriculus strips in a concentration-dependent manner. pEC_{50} values for the muscarinic agonists were different and the ranking order of pEC_{50} was as follows: (+) muscarine (7.81) >APE (7.66) > oxotremorine M (7.45) > carbachol (7.29) > methacholine (7.18) > arecoline (6.82) > acetylcholine (6.71) > bethanechol (5.81) =McN-A343 (5.79) =

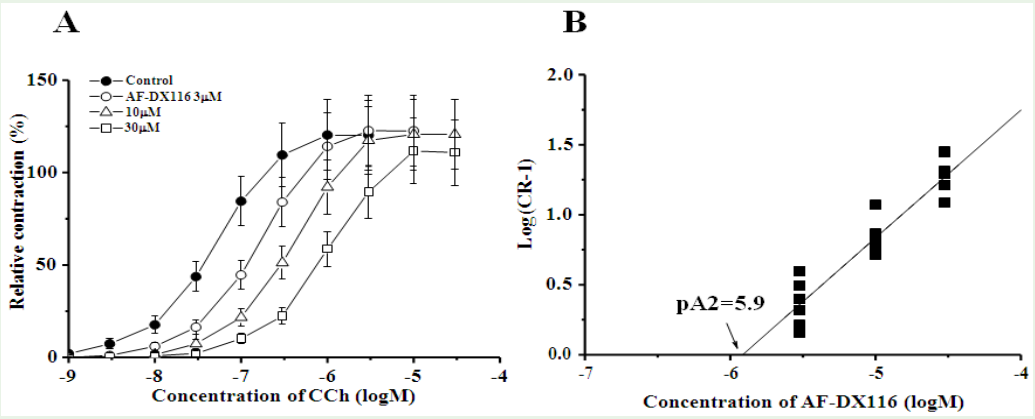

Figure 2: Inhibition of carbachol-induced contraction by AF-DX116 in the chicken proventriculus.
(A) Cumulative concentration-response curves for carbachol in the absence (●) and presence of increasing concentrations of AF-DX116 (○:3μM;Δ:10μM and □:30μM). (B) Schild plots for antagonism of carbachol-induced contraction by AF-DX116. pK_b was estimated to be 5.9. Values are means±S.E.M. of more than 5 experiments. Individual data were plotted in (B) and the regression line was drawn by computer analysis.

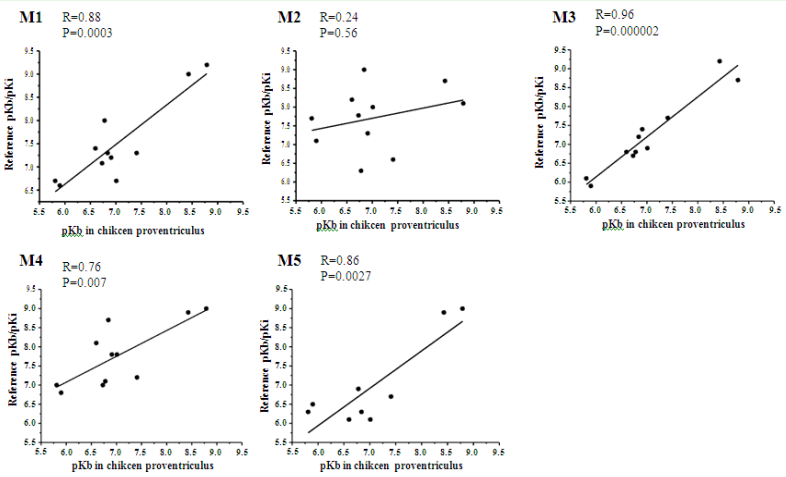

Figure 3: Comparison of muscarinic receptor antagonist affinities for determination of the receptor subtype in the chicken proventriculus. The x-axis represents the dissociation constants (pK_b) obtained in the contraction study of the proventriculus. The y-axis represents the mean value from the range of published pK_i/pK_b values for each antagonist in respective receptor subtypes (M1-M5, Table 1). The line represents the regression line. The correlation coefficient (R) and probability (P) are indicated in each figure.

pilocarpine (5.79). The maximum contractions of all muscarinic agonists were almost the same, indicating that all agonists act as full agonists. Figure 1 shows the correlation of pEC_{50} values for eight muscarinic receptor agonists with those values obtained in the guinea-pig ileum [30]. The correlation coefficient and probability were 0.88 and 0.0037, respectively and the slope was 1.007.

Effects of muscarinic receptor antagonists on carbachol-induced contraction

To identify muscarinic receptor subtype, the affinity for muscarinic receptor antagonists was determined by Schilds plot analysis. In antagonist experiments, carbachol was used as an agonist to cause contraction due to its insusceptibility to degradation by cholinesterase. Figure 2 shows the effects of three concentrations of AF-DX116 (3,10 and 30μM) on the concentration-response curves for carbachol. AF-DX116 shifted the concentration-response curve to the right in a concentration-dependent manner. The pEC_{50} values for carbachol were estimated to be 6.79±0.04 (3 μM), 6.43±0.06 (10 μM) and 6.02±0.05 (30 μM), and the pK_b value was calculated to be 5.90±0.08. 4-DAMP (10-100 nM), atropine (30-300 nM), p-F-HHSID (30-300 nM), himbacine (100 nM- 1 μM), pirenzepine (300 nM- 3 μM), AQ-RA741(1-10 μM), AF-DX384 (300 nM-3 μM), tropicamide (300 nM- 3 μM), dimethindene (1-10 μM) and methoctramine (3-30 μM) also inhibited the carbachol-induced contraction and caused a parallel rightward shift of the concentration-response curve. The estimated pK_b values and slope of Schild plots line were 8.79±0.05 and 1.11±0.02 (n=6) for 4-DAMP, 8.43±0.15 and 1.16±0.08 (n=6) for atropine, 7.41±0.08 and 1.10±0.10 (n=5) for p-F-HHSID, 7.01±0.08 and 0.91±0.07 (n=6) for himbacine, 6.78±0.06 and 0.96±0.06 (n=6) for pirenzepine, 6.60±0.11 and 1.05±0.03 (n=5) for AQ-RA741,

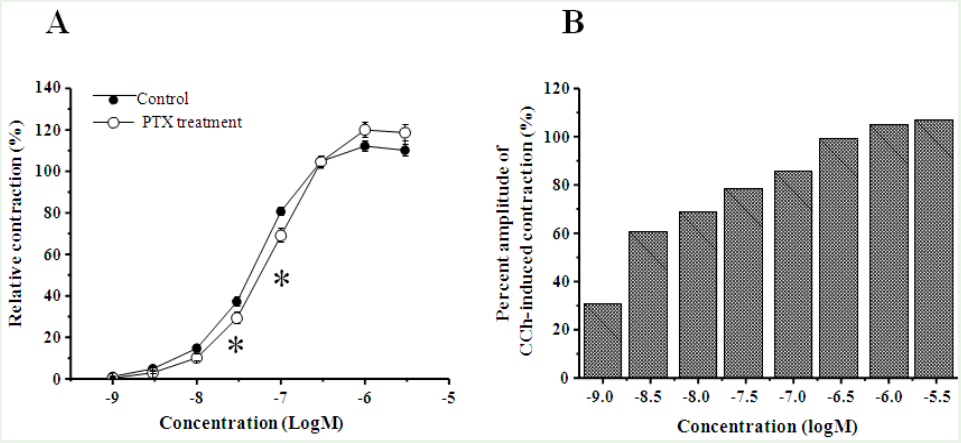

Figure 4: Effects of pertussis toxin treatment on contractile responses to carbachol in the proventriculus of chicken. Chickens were treated with an intraperitoneal injection of 100μg/kg of pertussis toxin approximately 72h before the study. (A) Concentration-response curves for carbachol were compared between control and pertussis toxin-treated chickens. Values are means±S.E.M. of at least 5 muscle strips isolated from 5 different chickens.
* The response was significantly different from that of the control. (B) Relative amplitude of carbachol-induced contraction in the proventriculus of chicken treated with pertussis toxin. Each column indicates percentage ratio of carbachol-induced contraction (100 x average contraction in the pertussis toxin treated chicken/ average contraction in normal condition).

6.84±0.05 and 1.07±0.03 (n=5) for AF-DX384, 6.91±0.08 and 1.04±0.07 (n=4) for tropicamide, 6.73±0.15, 0.65±0.08 (n=5) for dimethindene and 5.81±0.06, 1.00±0.06 (n=5) for methoctramine. Comparison of antagonist data for the chicken proventriculus with the mean published pK_i /pK_b values of the antagonists for a specific mammalian muscarinic receptor subtype (Table 1) was carried out to determine the muscarinic receptor subtype involved in the contractile response in the chicken proventriculus. An excellent correlation (r=0.96, P=0.000002, slope of regression line=1.06±0.1) was found by comparing the antagonist affinities from the chicken proventriculus with respective pK_b/ pK_i values for M_3 muscarinic receptor (Table 1, Figure 3). Correlations of pK_b values with other muscarinic receptor subtypes were also analyzed. Although there were good correlations between affinity data of the proventriculus and reference affinity values of M_1 (r=0.88, p=0.0003, slope=0.85±0.15), M_4 (r=0.76, P=0.007, slope=0.67±0.19) and M_5 (r=0.86, P=0.0027, slope=0.97±0.2) receptors, the correlation of antagonist pK_b values obtained in the proventriclus with reference affinity values for the M_2 receptor was not significant (r=0.24, P=0.562, slope=0.27±0.28) (Figure 3).

Effect of PTX on carbachol-induced contraction

The contractile responses to carbachol in proventriculus strips from PTX-pretreated chickens were lower than those of muscle strips from non-treated control chickens and the pEC_{50} value decreased significantly (Control: 7.29±0.02, n=17; PTX: 7.11±0.04, n=13), but the maximum contractions were not significantly different (Control: 112±2.6%, n=17; PTX: 120±3.5%, n=13) (Figure 4A). Inhibition of carbachol-induced contraction by PTX was 39% at 3 nM, 31% at 10 nM, 21% at 30 nM, 14% at 100 nM and 0.2% at 300 nM, indicating that the response to a low concentration of carbachol was sensitive to PTX treatment (Figure 4B). AF-DX116 (10μM) shifted the concentration-response curve for carbachol in proventriculus strips of PTX-pretreated chickens, and the pK_b value was calculated to be 5.86±0.06 (n=6).

Contractile responses to oxotremorine M in 4-DAMP mustard and AF-DX116-treated proventriculus preparations

In normal conditions, oxotremorine M (1 nM- 30 μM) caused contraction of the proventriculus (pEC_{50}=7.45). Pretreatment with AF-DX116 decreased this contraction and the estimated pK_b value was 5.78±0.11 (n=5). In the proventriculus preparations pretreated with 4-DAMP mustard and AF-DX116 (see Materials and Methods), oxotremorine M caused contraction of the proventriculus in the presence of 5-hydroxytryptamine and forskolin (pEC_{50}=5.85±0.15, n=5, 40-fold lower than that of the control). AF-DX116 inhibited the oxotremorine M-induced contraction and the obtained pK_b value (6.22±0.09, n=6) was significantly higher than that obtained in the normal condion, indicating an obvious increase in pK_b values.

[^{3}H]-QNB binding study

As shown in Figure 5, specific binding of [^{3}H]-QNB to the crude membrane increased with increment of the free concentration of the ligand and reached a plateau at 1.5-3.1 nM. Scatchard plots of saturation binding fitted a straight line and the estimated K_d and Bmax values were 0.17±0.03 nM and 560±70 fmol mg^{-1} protein (n=4), respectively. The muscarinic receptor antagonists inhibited the [^{3}H]-QNB binding in a concentration-dependent manner. Displacement curves were analyzed by both one-site and two-sites models using Graph pad Prism 4, but the fitting was not notably different between the two models. Therefore, we used the one-site model to calculate IC_{50} values and pK_i values. The mean pK_i values of muscarinic receptor antagonists were 8.47 for atropine, 7.77 for 4-DAMP, 5.9 for AF-DX116, 7.8 for AF-DX384, 7.22 for pirenzepine, 7.1 for tropicamide, 7.0 for AQ-RA741, 6.9 for himbacine, 6.9 for methoctramine, 6.72 for p-F-HHSID and 6.59 for dimethindene. The obtained pK_i values were significantly correlated with pK_b values of the antagonists in the contraction study (r=0.73, P=0.01, slope=0.97±0.3). The pK_i values of the chicken proventriculus were compared with the reference pK_i/pK_b values for respective muscarinic receptor subtypes (Table 1). Correlation coefficients and probabilities were high for the M_1 (r=0.78, p=0.004), M_3 (r=0.81, p=0.002) and M_4 (r=0.86, p=0.008) receptor subtypes, slightly high for the M_5 (r=0.67, p=0.048) and low for the M_2 receptor (r=0.58, p= 0.057).

Cyclic AMP study

Resting cyclic AMP content of the proventriculus was 17.7±1.3 pmol/mg protein (n=4). Isoproterenol (100 nM-10 μM) increased the cyclic AMP content in a concentration-dependent manner (100 nM: 77.4±19.8 pmol/mg protein n=6; 1 μM: 102.8±11.5pmol/mg protein n=6; 10μM: 125±19.0 pmol/mg protein n=6). Pretreatment with carbachol (1, 10 and 100 μM) significantly decreased the tissue cyclic AMP contents increased by 1μM isoproterenol (1μM: 38.4±6.1 pmol/mg protein n=6; 10μM: 27±5.9 pmol/mg protein n=5; 100μM: 16.1±4.3 pmol/mg protein n=6).

Discussion

Acetylcholine is a neurotransmitter of parasympathetic postganglionic nerves (cholinergic nerves) and causes contraction of gastrointestinal smooth muscle by activation of muscarinic receptors. In the gastrointestinal smooth muscle of some mammals, it has been reported that the M_3 receptor coupled to phospholipase C mediates most of the contraction by muscarinic receptor agonists [3,6,10-12]. However, many studies (competitive radioligand binding, immunoprecipitation and molecular biological studies) have demonstrated the coexistence of muscarinic M_2 and M_3 receptors [3,6,31]. The role of the M_2 recptor (inhibition of adenylate cyclase activity) in muscarinic smooth muscle contraction has been investigated. Under experimental conditions in which intracellular cyclic AMP is elevated by forskolin or isoproterenol, M_2 receptor stimulation can attenuate cyclic AMP production and cause contraction indirectly [6,25]. However, in studies using muscarinic receptor knockout mice, M_2 or M_3 receptor excitation caused gastrointestinal contraction individually, though pharmacological properties of the carbachol-induced contraction in wild-type mice indicated the nature of M_3 receptor [15-17]. Synergic action between M_2 and M_3 receptors has been demonstrated, and simultaneous excitation of both receptors can cause relatively large gastrointestinal contraction from a low agonist concentration [32,33]. Muscarinic agonists also cause contraction of gastrointestinal tract of chickens, bullfrogs and goldfish, and the non-selective muscarinic receptor antagonist atropine decreased the responses to muscarinic agonists, indicating the involvement of muscarinic receptors [18-20]. However muscarinic receptor subtype in the gastrointestinal tract of the non-mammalian vertebrates have not been characterized in detail despite the phylogenic interest of muscarinic receptor sub-

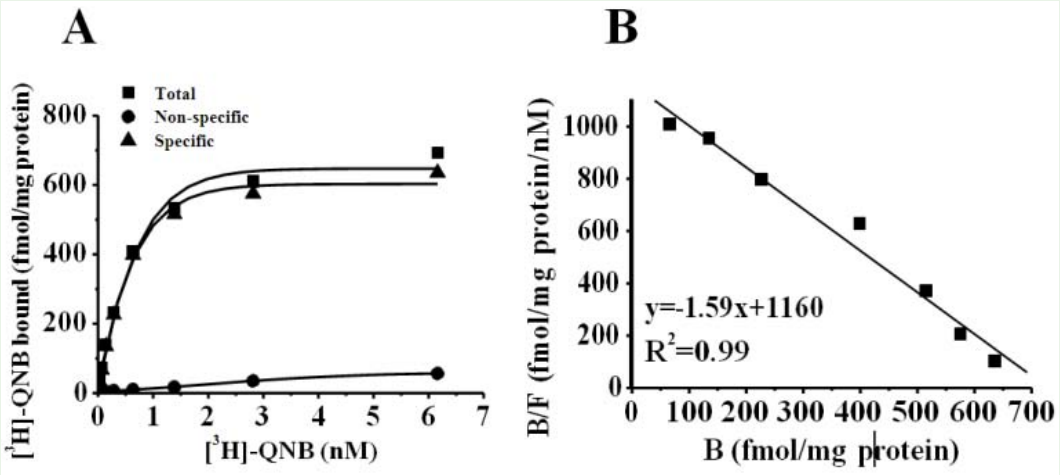

Figure 5: Binding of [^{3}H]-QNB to the homogenate of the chicken proventriculus.
(A) Crude membrane preparations obtained from the chicken proventriculus were incubated with increasing concentrations of [^{3}H]-QNB for 60 min at 37°C. Specific binding was determined as the difference between total binding and non-specific binding obtained in the presence of 100 μM atropine. Abscissa: [^{3}H]-QNB concentration (nM). Ordinate: Specific [^{3}H]-QNB bound (fmol mg^{-1} protein). (B) Scatchard plot of the binding data. The line was determined by linear regression analysis. The points shown are from one of 4 experiments.

types for cholinergic regulation of gastrointestinal tract. In the present experiments, we examined the pharmacological and biochemical characteristics of muscarinic receptor agonist-induced contraction in the chicken proventriculus (stomach) and showed that the M_3 type muscarinic receptor is mainly involved in the muscarinic contraction, and the possibility of contribution of muscarinic receptor linked cyclic AMP decrease (M_2 or M_4 receptor) cannot be excluded.

Carbachol caused the hexamethonium- or tetrodotoxin-insensitive but atropine-sensitive contractions in the chicken proventriculus, indicating that the muscarinic receptor causing contraction is located on smooth muscle cells. The significant correlation of agonistic affinity (pEC_{50}) between the chicken proventriculus and guinea-pig ileum (M_3 receptor having been demonstrated to mediate contraction, Eltze et al. [30]) suggested that M_3 type receptor mediates the muscarinic agonist-induced contraction of the chicken proventriculus. Involvement of the M_3 receptor was further supported by the results of a competitive study of carbachol-induced contraction using muscarinic receptor antagonists. The eleven muscarinic receptor antagonists shifted the concentration-response curve for carbachol in the rightward direction without decreasing the maximum contraction. The calculated pK_b values were compared with the reference affinity values pK_b/pK_i and the correlation coefficient, probability and slopes were estimated. Tietje and Nathanson [34] and Creason et al. [35] reported high homology and similar pharmacological properties of chicken M_2 and M_5 muscarinic receptors with respective mammalian muscarinic receptor subtypes. Therefore, the affinity of muscarinic receptor antagonists is thought not to be markedly different between mammals and chickens. Among five muscarinic receptor subtypes, the highest and significant correlation was found between mammalian M_3 subtype and the chicken muscarinic receptor involved in the contraction. In addition, the slope of the regression line was almost 1.0. These results indicated that the M_3 subtype is involved in the muscarinic contraction in the chicken proventriculus, similar with the case of the chicken ileum [23] and mammalian gastrointestinal tract [3,6,10-12].

Scatchard plots of saturation binding of [^{3}H]-QNB fitted a straight line and the estimated K_b value was 0.17 nM. This value is almost consistent with the values of [^{3}H]-QNB binding in the rat ileum (0.08-0.15 nM, Michalek et al., [36]), guinea-pig caecum (0.18 nM, Mita and Uchida, [37]) and chicken ileum (0.16 nM, Darroch et al. [23]). pK_i values for muscarinic antagonists obtained from the displacement study correlated with M_1, M_3 and M_4 receptors subtypes not with the M_2 receptor subtype, being slightly different from the results obtained in the present contraction studies. Darroch et al. also investigated the muscarinic receptor subtypes in the chicken ileum by contraction and binding displacement studies and they found different profiles of muscarinic receptors in the contraction study (M_3 type) and binding study (M_2 type) [23]. In the present study, the correlation of pK_i was quite different from that of the chicken ileum, and the results suggested the possible involvement of M_3 type receptor in the muscarinic contraction of chicken proventriculus. The reasons for different binding characteristics in the proventriculus and ileum are not known in the experiment, but heterogeneous expression of M_2 muscarinic receptors in the proventriculus and ileum (ileum > proventriculus) might be one reason.

To determine the involvement of a non-M_3 receptor subtype in the carbachol-induced contraction, we examined the effects of PTX, which causes dysfunction of M_2/M_4- receptor linked signal transduction acting on the Gi protein [15,25]. In proventriculus strips isolated from PTX-treated chickens, the concentration-response curve for carbachol was slightly but significantly shifted to the rightward direction, resulting in a decrease in the pEC_{50} value. Inhibition by PTX treatment was marked at low concentrations as reported in the mouse ileum [15]. The results suggest the involvement of PTX-sensitive muscarinic receptors such as M_2 and M_4 receptors in the part of the carbachol-induced contraction, probably by a low concentration of carbachol. The presence of M_2/M_4 muscarinic receptors in the proventriculus is supported by the results of the cyclic AMP study showing that the carbachol-induced decrease of tissue cyclic AMP content elevated by isoproterenol. M_2 and M_4 receptors

are coupled with the Gi protein, and activation of these receptors causes a decrease in cyclic AMP content.

4-DAMP mustard caused irreversible muscarinic receptor alkylation in the isolated gastrointestinal tract. Because the affinity of 4-DAMP for the M_3 receptor is 10-times higher than that for the M_2 receptor and the affinity of AF-DX116 for the M_2 receptor is 15-times higher than that for the M_3 receptor (Table 1), pretreatment with appropriate concentrations of 4-DAMP mustard and AF-DX116 can preferentially cause irreversible alkylation of a non-M_2 receptors (M_2 receptor alkylation was protected by AF-DX116). After washing out both antagonists, oxotremorine M caused contraction in the 5-hydroxytryptamine and forskolin-pretreated preparation through inhibition of adenylate cyclase, which is downstream of M_2 receptor activation. In the present study, pK_b value of AF-DX116 (6.22) for the oxotremorine M-induced contraction in 4-DAMP mustard and AF-DX116- treated preparations was significantly higher than the affinity for the M_3 receptor (5.78). The reason for increase of pK_b value is suggested to be increase in the contribution of M_2 type receptor in the contractile responses to oxotremorine M probably due to alkylation of non-M_2 receptors. The results also support the presence of the M_2 receptor in the chicken proventriculus. Although possible presence of M_2 receptor in the proventriculus, it was not able to detect M_2 receptor profiles in binding studies. The reason for this was not clarified in the present study. However, in the mammalian gastrointestinal tract, the ratio of M_2:M_3 receptors has been demonstrated to be 3-5:1 and the binding data fit the two-site model. In the chicken proventriculus, the difference between the one-site and two-site binding model was not so marked, suggesting that although the M_2 receptor is present in the proventriculus, most of the muscarinic receptors are the M_3 receptor subtype, unlike in the case of the mammalian gastrointestinal tract.

In conclusion, a muscarinic agonist mainly acts on the M_3 type receptor to cause contraction of the chicken proventriculus, but the possible involvement of M_2/M_4 type receptors cannot be excluded at present. Since M_3 receptor is common subtype mediating the muscarinic contraction of gastrointestinal tract, it might be interesting to examine whether the M_3 type muscarinic receptor is involved in muscarinic contraction in lower vertebrates such as fish and amphibians. However, since the selectivity of muscarinic receptor agonists and antagonists is limited and there might be some difference in the structures of muscarinic receptor, a molecular biological study to determine structure and expression of muscarinic receptor mRNAs will be needed for comparative pharmacology of muscarinic receptor subtypes in the gastrointestinal tract.

References

1. Peralta EG, Ashkenazi A, Winslow JW, Smith DH, Ramachandran J. Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. EMBO J. 1987; 6: 3923-3929.
2. Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev. 1998; 50: 279-290.
3. Eglen RM, Hegde SS, Watson N. Muscarinic receptor subtypes and smooth muscle function. Pharmacol Rev. 1996; 48: 531-565.
4. Lanzafame AA, Christopoulos A, Mitchelson F. Cellular signaling mechanisms for muscarinic acetylcholine receptors. Receptors Channels. 2003; 9: 241-260.
5. Ehlert FJ, Pak KJ, Griffin MT. Muscarinic agonists and antagonists: effects on gastrointestinal function. Handb Exp Pharmacol. 2012; 208: 343-374.
6. Ehlert FJ. Pharmacological analysis of the contractile role of M_2 and M_3 muscarinic receptors in smooth muscle. Receptors Channels. 2003; 9: 261-277.
7. Giraldo E, Micheletti R, Montagna E, Giachetti A, Vigano MA, et al. Binding and functional characterization of the cardio-selective muscarinic antagonist methoctramine. J. Pharmacol. Exp. Ther. 1988; 244: 1016-1020.
8. Maeda A, Kubo T, Mishina M, Numa S. Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. FEBS Lett. 1988; 239: 339-342.
9. Wall SJ, Yasuda RP, Li M, Ciesla W, Wolfe BB. Differential regulation of subtype's m_1-m_5 of muscarinic receptors in forebrain by chronic atropine administration. J. Pharmacol. Exp. Ther. 1992; 262: 584-588.
10. Honda K, TakanoY, Kamiya H. Pharmacological profiles of muscarinic receptors in the longitudinal smooth muscle of the guinea-pig ileum. Jpn. J. Pharmacol. 1993; 62: 43-47.
11. Kerr P, Hillier K, Wallis R, Garland C. Characterization of muscarinic receptors mediating contractions of circular and longitudinal muscle of human isolated colon. Br.J.Pharmacol. 1995; 115: 1518-1524.
12. Leclere PG, Lefebvre RA. Characterization of pre- and postsynaptic muscarinic receptors in circular muscle of pig gastric fundus. Br. J. Pharmacol. 2002; 135: 1245-1254.
13. Thomas EA, Baker SA, Ehlert FJ. Functional role for the M_2 muscarinic receptor in smooth muscle of guinea-pig ileum. Mol. Pharmacol. 1993; 44: 102-110.
14. Ehlert FJ, Griffin MT. The use of irreversible ligands to inactivate receptor subtypes: 4-DAMP mustard and muscarinic receptors in smooth muscle. Life Sci. 1998; 62: 1659-1664.
15. Unno T, Matsuyama H, Sakamoto T, Uchiyama M, Izumi Y, et al. M_2 and M_3 muscarinic receptors mediated contractions in longitudinal smooth muscle of the ileum studied with receptor knockout mice. Br. J. Pharmacol. 2005; 146: 98-108.
16. Kitazawa T, Hashiba K, Cao J, Unno T, Komori S, et al. Functional roles of muscarinic M_2 and M_3 receptors in mouse stomach motility: Studies with muscarinic receptor knockout mice. Eur. J. Pharmacol. 2007; 554: 212-222.
17. Tanahashi Y, Waki N, Unno T, Matsuyama H, Iino S, et al. Roles of M_2 and M_3 muscarinic receptors in the generation of rhythmic motor activity in mouse small intestine. Neurogastroenterol Motil. 2013; 25: 687-697.
18. Kitazawa T, Taneike T, Ohga A. Functional characterization of neural and smooth muscle motilin receptors in the chicken proventriculus and ileum. Regul Pept. 1997; 71: 87-95.
19. Kitazawa T, Itoh K, Yaosaka N, Maruyama K, Matsuda K, et al. Ghrelin does not affect gastrointestinal motility in rainbow trout and goldfish in vitro. Gen. Comp. Endocrinol. 2012; 178: 539-545.
20. Kitazawa T, Shimazaki M, Kikuta A, Yaosaka N, Teraoka H, et al. Effects of ghrelin and motilin on smooth muscle contractility of the isolated gastrointestinal tract from the bullfrog and Japanese fire belly newt. Gen. Comp. Endocrinol. 2016; 232: 51-59.
21. Nouchi H, Kaeriyama S, Muramatsu A, Sato M, Hirose K, et al. Muscarinic receptor subtypes mediating positive and negative inotropy in the developing chick ventricle. J. Pharmacol. Sci. 2007; 103: 75-82.
22. Kitazawa T, Asakawa K, Nakamura T, Teraoka H, Unno T, et al. M_3 muscarinic receptors mediate positive inotropic responses in mouse atria: a study with muscarinic receptor knockout mice. J. Pharmacol .Exp. Ther. 2009; 330: 487-493.
23. Darroch S, Irving HR, Mitchelson FJ. Characterisation of muscarinic receptor subtypes in avian smooth muscle. Eur. J. Pharmacol. 2000; 402, 161-169.
24. Arunlakshana A, Schild H. Some quantitative uses of drug antagonists. Br. J. Pharmacol. 1959; 14: 48-57.
25. Thomas EA, Ehlert FJ. Pertussis-toxin blocks M_2 muscarinic receptor-mediated effects on contraction and cyclic AMP in the guinea-pig ileum, but

not M_3-mediated contractions and phosphoinositide hydrolysis. J. Pharmacol. Exp. Ther. 1994; 271: 1042-1050.

26. Watson N, Reddy H, Eglen RM. Role of muscarinic M_2 and M_3 receptors in guinea-pig trachea: Effects of receptor alkylation. Eur. J. Pharmacol. 1995; 278: 195-201.

27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951; 193: 265-275.

28. Cheng Y, Prusoff WH. Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol. 1973; 22: 3099-3108.

29. Kitazawa T, Yamada Y, Iwano H, Yokota H, Yuasa A, et al. Smooth muscle layer-dependent distribution of 5-HT7 receptor in the porcine myometrium. Br. J. Pharmacol. 2000; 13: 79-89.

30. Eltze M, Konig H, Ullrich B, Grebe T. Contraction of guinea-pig gallbladder: Muscarinic M_3 or M_4 receptors? Eur. J. Pharmacol. 1997; 332: 77-87.

31. Levey AL. Immunological localization of m_1-m_5 muscarinic acetylcholine receptors in peripheral tissues and brain. Life Sci. 1993; 52: 441-448.

32. Sakamoto T, Unno T, Kitazawa T, Taneike T, Yamada M, et al. Three distinct muscarinic signaling pathways for cationic channel activation in mouse gut smooth muscle cells. J. Physiol. 2007; 582: 41-61.

33. Matsuyama H, TanahashiY, Kitazawa T, Yamada M, Komori S, et al. Evidence for M_2 and M_3 muscarinic receptor involvement in cholinergic excitatory junction potentials through synergistic activation of cation channels in the longitudinal muscle of mouse ileum. J Pharmacol Sci. 2013; 121: 227-236.

34. Tietje K, Nathanson N. Embryonic chick heart expresses multiple muscarinic acetylcholine receptor subtypes. Isolation and characterization of a gene encoding a novel m_2 muscarinic acetylcholine receptor with high affinity for pirenzepine. J. Biol. Chem. 1991; 266: 17382-17387.

35. Creason S, Tietje KM, Nathanson NM. Isolation and functional characterization of the chick M_5 muscarinic acetylcholine receptor gene. J. Neurochem. 2000; 74: 882-885.

36. Michalek H, Fortuna S, Pintor A. Age-related changes in muscarinic receptor and post-receptor mechanisms in brain and ileum strip of rats. Acta Neurobiol Exp (Wars). 1993; 53: 93-101.

37. Mita M, Uchida MK. Muscarinic receptor binding and Ca^{2+} influx in the all-or-none response to acetylcholine of isolated smooth muscle cells. Eur. J. Pharmacol. 1988; 151: 9-17.

38. Bonner T, Young A, Brann M, Buckley N. Cloning and expression of the human and rat m_5 muscarinic acetylcholine receptor genes. Neuron. 1988; 1: 403-410.

39. Gardner A, Darroch S, Choo LK, Mitchelson F. The effects of some selective agonists and antagonists on peripheral muscarinic receptors. Trends Pharmacol. Sci. 1988; 40-43.

40. Buckley N, Bonner T, Buckley C, Brann M. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K_1 cells. Mol. Pharmacol. 1989; 35: 469-476.

41. Giraldo E, Vigano M, Hammer R, Ladinsky H. Characterization of muscarinic receptors in guinea pig ileum longitudinal smooth muscle. Mol. Pharmacol. 1988; 33: 617-625.

42. Eglen R, Whiting R. Competitive and non-competitive antagonism exhibited by 'selective' antagonists at a trial and ileal muscarinic receptor subtypes. Br. J. Pharmacol. 1987; 90: 701-707.

43. Waelbroeck M, Tastenoy M, Camus J, Christophe J. Binding of selective antagonists to four muscarinic receptors M_1 to M_4 in rat forebrain. Mol. Pharmacol. 1990; 38: 267-273.

44. Dorje F, Friebe T, Tacke R, Mutschler E, Lambrecht G. Novel pharmacological profile of muscarinic receptors mediating contraction of the guinea-pig uterus. Naunyn-Schmeideberg's Arch. Pharmacol. 1990; 342: 284-289.

45. Dorje F, Wess J, Lambrecht G, Tacke R, Mutschler E, et al. Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J. Pharmacol. Exp. Ther. 1991; 256: 727-733.

46. Liebmann C, Nawrath S, Schnitter M, Schubert H, Jakobs KH. Binding characteristics and functional G protein coupling of muscarinic acetylcholine receptors in rat duodenum smooth muscle membranes. Naunyn-Schmiedeberg's Arch. Pharmacol. 1992; 345: 7-15.

47. Lazareno S, Birdsall NJM. Pharmacological characterization of acetylcholine-stimulated [35s]-GTP gammas binding mediated by human muscarinic m_1-m_4 receptors: antagonist studies. Br. J. Pharmacol. 1993; 109: 1120-1127.

48. Doods H, Entzeroth M, Ziegler H, Mayer N, Holzer P. Pharmacological profile of selective muscarinic receptor antagonists on guinea-pig ileal smooth muscle. Eur. J. Pharmacol. 1994; 253: 275-281.

49. Eglen RM, Reddy H, Watson N. Selective inactivation of muscarinic receptor subtypes. Int. J. Biochem. 1994; 26: 1357-1368.

50. Pfaff O, Hildebrandt C, Waelbroeck M, Hou X, Moser U, et al. The (S)-(+)-enantiomer of dimethindene: A novel M_2-selective mACh antagonist. Eur. J. Pharmacol. 1995; 286: 229-240.

51. Melchiorre C, Minarini A, Budriesi R, Chiarini A, Spampinato S, et al. The design of novel methoctramine-related tetra amines as muscarinic receptor subtype selective antagonists. Life Sci. 1995; 56: 837-844.

52. Esqueda E, Gerstin E, Griffin M, Ehlert F. Stimulation of cyclic AMP accumulation and phosphoinositide hydrolysis by M_3 muscarinic receptors in the rat peripheral lung. Biochem. Pharmacol. 1996; 52: 643-658.

53. Shi H, Wang h, Wang Z. Identification and characterization of multiple subtypes of muscarinic acetylcholine receptors and their physiological functions in canine hearts. Mol. Pharmacol. 1999; 55: 497-507.

54. Eglen RM, Nahorski SR. The muscarinic m_5 receptor: A silent or emerging subtype? Br. J. Pharmacol. 2000; 130: 13-21.

55. Nasman J, Jolkkonen M, Ammoun S, karlsson E, Akerman KE. Recombinant expression of a selective blocker of M_1 muscarinic receptors. Biochem. Biophys. Res. Commun. 2000; 271: 435-439.

56. Wang Z, Shi H, Wang H. Functional M_3 muscarinic acetylcholine receptors in mammalian hearts. Br. J. Pharmacol. 2004; 142: 395-408.

57. Lazareno S, Buckey N, Roberts F. Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. Mol. Pharmacol. 1990; 38: 805-815.