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Characterization of the muscarinic receptor subtype that mediates the contractile response of acetylcholine in the swine myometrium

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Abstract

The aim of the present study was to characterize the subtype of muscarinic receptor that mediates acetylcholine-induced contractions in the nonpregnant proestrus swine myometrium by means of mechanical, radioligand ([^{3}H]quinuclidinyl benzilate) binding and biochemical (measurement of cyclic AMP) approaches. Acetylcholine ($-\log EC_{50}$, 6.12), oxotremorine-methiodide (6.47), methacholine (6.35), carbachol (6.18) and muscarine (6.33) caused contractile responses of the uterine circular muscle, with a similar maximum amplitude, but pilocarpine and McN-A-343 (4-(*m*-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium) were ineffective in causing contraction. The contractile response to acetylcholine was antagonized by the following muscarinic receptor antagonists in a competitive manner (with pA_2 values in parentheses): atropine (8.95), 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP, 8.83), tropicamide (7.07), himbacine (7.01), pirenzepine (6.42) and 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3*b*][1,4]benzodiazepin-6-one (AF-DX116, 5.96). Electrical field stimulation (10 Hz) caused tetrodotoxin- and atropine-sensitive contractions in the circular muscle. All muscarinic receptor antagonists decreased the electrical field stimulation-induced contraction in a concentration-dependent manner. The order of inhibition ($-\log IC_{50}$) was 4-DAMP (8.35) > tropicamide (6.72) > himbacine (6.54) > pirenzepine (6.31) > AF-DX116 (6.13). Acetylcholine did not affect the cytoplasmic cyclic AMP level, regardless of the presence or absence of forskolin, suggesting the absence of functional muscarinic M_2 and/or M_4 receptors in the swine myometrium. The receptor binding study indicated that circular muscle layers of the swine myometrium contained a single class of [^{3}H]quinuclidinyl benzilate binding site ($K_d = 0.92$ nM; $B_{max} = 126.6$ fmol/mg protein). Specific binding was displaced by muscarinic receptor antagonists in the following order (with pK_i value and Hill coefficient in parentheses): atropine (8.22 and 0.93) > 4-DAMP (8.18 and 0.94) > tropicamide (6.78 and 0.93) > pirenzepine (5.46 and 0.92) > AF-DX116 (5.12 and 0.94). The present results suggest that in circular muscle layers of the swine myometrium, exogenous and endogenous acetylcholine cause contraction through activation of muscarinic M_3 receptors present on smooth muscle cells.

Keywords: Myometrium (swine); Acetylcholine; Muscarinic M_3 receptor; Cholinergic nerve; cAMP

1. Introduction

The heterogeneity of muscarinic acetylcholine receptors belonging to the superfamily of G-protein-coupled receptors is well established. At present, muscarinic receptors have been classified by pharmacological and signal transductional criteria into four major subtypes: M_1 (coupled to the stimulation of phosphoinositide hydrolysis), M_2 (linked to the inhibition of adenylate cyclase), M_3 (coupled to the stimulation of phosphoinositide hydrolysis) and M_4 (linked to the inhibition of adenylate cyclase). The functional properties and binding profiles of the muscarinic M_1, M_2, M_3 and M_4 receptors closely correspond to those of the m_1, m_2, m_3 and m_4 receptor subtypes, which have been identified in recent receptor cloning studies. Each receptor subtype has been shown to be distributed in a tissue-selective manner; e.g., the M_1 type is distributed in the central nervous system and autonomic ganglia, the M_2 type is distributed in cardiac muscle, the M_2 and M_3 types are distributed in visceral smooth muscle, and the M_4 type is distributed in the lung. In addition, recent molecular cloning studies have indicated the presence of one (m_5) less well-defined (in functional terms) subtype whose signal transductional mechanism is the same as that of mus-

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carinic M_1 and M_3 receptors (positively coupled to phospholipase C/phosphoinositide hydrolysis) (Hulme et al., 1990; Caulfield, 1993; Levey, 1993; Eglen et al., 1996). Because there is a lack of selective agonists for each muscarinic receptor subtype, and the absolute selectivity of currently available muscarinic receptor antagonists for any one muscarinic receptor is poor, muscarinic receptors can be separated pharmacologically by the rank order of several antagonists. The rank order of antagonist affinity for muscarinic receptor subtypes is 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) > pirenzepine > darifenacin > himbacine > methoctramine at the M_1 type; 4-DAMP > himbacine > methoctramine > 11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3*b*][1,4]benzodiazepin-6-one (AF-DX116) > pirenzepine at the M_2 type; darifenacin > 4-DAMP > himbacine > pirenzepine > AF-DX116 at the M_3 type; and 4-DAMP > himbacine > tropicamide > pirenzepine > AF-DX116 at the M_4 type (Lazerano and Birdsall, 1993; Doods et al., 1993, Eglen et al., 1996). Concerning the muscarinic m_5 receptor, although there is no single ligand that preferentially acts on the protein expressed by the m_5 gene and there have been no functional studies, the pharmacological profile (rank order of antagonists) of the m_5 receptor differs from that of other muscarinic receptors (Hulme et al., 1990; Dörje et al., 1991; Eglen et al., 1996).

It is generally accepted that uterine activity is regulated by complex and mutual interactions among sex hormones, myometrial contractility and neurotransmitters. Functional studies with electrical field stimulation and extrinsic nerve stimulation have provided evidence of excitatory cholinergic as well as excitatory (α-adrenoceptor) and/or inhibitory (β-adrenoceptor) adrenergic innervation of the uterus (Nakanishi and Wood, 1971; Morizaki et al., 1989; Sato et al., 1989; Taneike et al., 1991, 1994). Although atropine-sensitive cholinergic contractions would suggest the presence of muscarinic receptors, the receptor subtype that mediates contraction of the rat, rabbit and human myometrium has not been well defined yet (Eglen et al., 1996; Eglen and Hegde, 1997). In the guinea-pig myometrial membrane, the muscarinic M_2 receptor was the dominant muscarinic receptor subtype detected in a radioligand binding study (Doods et al., 1993), and this type (M_2) was demonstrated to be functional (Eglen et al., 1989). However, the involvement of muscarinic M_3 and M_4 receptors in contractile responses of the guinea-pig uterus elicited by muscarinic receptor agonists has also been suggested (Dörje et al., 1990; Leiber et al., 1990). Owing to the limited number of studies on muscarinic receptor subtypes mediating the contraction of uterine smooth muscles, species- and estrous cycle-related variations in functional muscarinic receptors are not clearly understood at the present time. In a previous paper, we reported muscle layer-related differences in autonomic innervation of the swine myometrium; i.e., adrenergic predominance in longitudinal muscles and exclusive cholinergic innervation in circular muscles. Atropine-sensitive responses in the circular muscle and specific binding sites for [^{3}H]quinuclidinyl benzilate in the myometrial membrane indicated the presence of muscarinic receptors in the swine uterus (Taneike et al., 1991, 1994), but the receptor subtype has not yet been identified.

In the present series of experiments, in order to characterize the muscarinic receptor subtype that mediates the contractile responses to acetylcholine of the nonpregnant proestrus swine uterus, circular muscle strips were isolated and used for the following in vitro experiments: comparison of muscarinic receptor agonist responsiveness (EC_{50}), evaluation of muscarinic receptor antagonist affinity (pA_2) against acetylcholine-induced contractions, the effects of muscarinic receptor antagonists on electrical field stimulation-induced contraction (cholinergic, Taneike et al., 1994), [^{3}H]quinuclidinyl benzilate binding in myometrial membranes and displacement by antagonists, and the effects of acetylcholine on tissue cyclic AMP production in the myometrium.

2. Materials and methods

2.1. Tissue preparations

The animals used in the present study were 80 sexually mature, crossbred virgin gilts (6–8 months old). Fresh uteri with intact ovaries were provided by a local abattoir and were used for experiments on the day of slaughter. Only the uteri of pigs judged to be in proestrus, based on gross examination of the follicle size and appearance of the corpora lutea, were used (McDonald, 1975). Circular muscle layers were prepared surgically from the antimesometrial coat of the adtubal region (10 cm distal from the apex) in either left or right cornu by surgical procedures described previously (Taneike et al., 1994; Kitazawa et al., 1997). In brief, after removal of the endometrium, the muscle coat parallel to the direction of the circular muscle fiber was isolated. The longitudinal muscle layers were then removed from each muscle segment by meticulously cutting them away with fine scissors under a binocular microscope, thereby isolating the remaining circular muscle for experimental use.

Circular muscle strips (10×1 mm) were suspended vertically in an organ bath (20 ml) containing 37°C Krebs solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11.5 mM glucose) bubbled with 95% O_2 + 5% CO_2. A force-displacement transducer (SB-1T, Nihon Kohden) equipped with a pen-writing recorder (Recticorder, Nihon Kohden) was used to measure the mechanical activity of the myometrial preparations. The muscle strips were loaded at 0.2 g and allowed to equilibrate for 60 min. During the equilibration period, each muscle preparation showed a spontaneous phasic contraction whose frequency was $11.2 \pm 0.6/5$ min ($n = 15$). These spontaneous contractions

were unaffected by atropine (1 μM) or tetrodotoxin (1 μM), indicating the myogenic origin of the contractions.

2.2. Experimental protocol and data analysis of contraction study

After steady spontaneous contractile activity was established, acetylcholine and muscarinic receptor agonists were applied cumulatively to the organ bath at 2 min intervals. The development of uterine muscle tension (elevation of tonus) was measured isometrically, and concentration–response curves were made (amplitude of contraction was normalized using 100 μM acetylcholine-induced response and expressed as a percentage). The EC_{50} values (concentration of agonists that caused half-maximum contraction) were determined by least-squares nonlinear regression analysis of each concentration–response curve.

Antagonist activity (pA_2) against the acetylcholine-induced contraction was determined for each muscarinic receptor antagonist (atropine, pirenzepine, AF-DX116, 4-DAMP, himbacine, tropicamide) according to the procedure of Arunlakshana and Schild (1959). Concentration–response curves for acetylcholine were made at 45-min intervals in the absence and presence (30-min pretreatment) of three increasing concentrations of the antagonists. On the basis of EC_{50} values with and without the antagonists, the concentration ratio (EC_{50} in the presence of antagonist/EC_{50} in the absence of antagonist) was determined for each antagonist. If the blockade was competitive under the equilibrium condition, then a plot of the logarithm of concentration ratio − 1 against the negative logarithm of the molar concentration of the antagonist should yield a straight line, whose slope is not different from unity (1.00) and whose intercept on the abscissa is the pA_2 value, which is generally considered to be equivalent to the apparent antagonist dissociation constant to receptors ($-\log K_b$).

The effects of muscarinic receptor antagonists on the response induced by endogenous acetylcholine were also examined. To excite cholinergic nerves in the wall of the circular muscles, electrical field stimulation was delivered to the muscle strips via a pair of platinum wire electrodes placed in parallel on each side of the preparation (8 mm in width). Repetitive impulses of 10 Hz, consisting of rectangular waves (supramaximum voltage, 50 V; duration, 0.6 ms), applied for 60 s caused reproducible contractile responses at 6-min intervals. After the control response to 10 Hz stimulation was obtained, three or four increasing concentrations of muscarinic receptor antagonists were applied every 15 min, and their effects on the electrical field stimulation-induced contractions were analyzed. The IC_{50} values (concentration of antagonists that caused 50% inhibition of the stimulation-induced response) of each muscarinic receptor antagonist were calculated from the concentration–inhibition relationships and compared among the antagonists.

2.3. Measurement of cyclic AMP

To investigate the inhibitory action of acetylcholine on adenylate cyclase activity, which is probably mediated by muscarinic M_2 and/or M_4 receptors, the effect of acetylcholine on cyclic AMP production was examined in the swine myometrium. Isolated fresh circular muscle strips weighing approximately 40–50 mg were incubated in Krebs solution 37°C for 1 h and then exposed to 3 μM acetylcholine for 1, 2 and 5 min. Strips just before application of acetylcholine were used as untreated controls (0 min). After incubation for the given times, the muscle strips were frozen quickly in liquid nitrogen and homogenized in 6% trichloroacetic acid solution with a Polytron. After centrifugation (3000 rpm, 2 times), the trichloroacetic acid in the supernatant was removed by washing with water-saturated ether, and cyclic AMP was assayed using an enzyme immunoassay kit (Amersham). The effects of acetylcholine and clonidine (α_2-adrenoceptor agonist) on cyclic AMP production stimulated by forskolin were also examined. The α_2-adrenoceptor is known to be negatively coupled with adenylate cyclase activity, as are muscarinic M_2 and M_4 receptors (Ruffolo et al., 1991), and is present in the swine myometrium (Taneike et al., 1995). Circular muscle strips were incubated with 3 μM forskolin for 5 min, and then acetylcholine (10 μM) or clonidine (10 μM) were applied to the bath with forskolin for 5 min, and finally the reaction was stopped by freezing the strips in liquid nitrogen. Cyclic AMP was extracted from the myometrial tissues and measured with an enzyme immunoassay. Tissue cyclic AMP levels are expressed as pmol/g tissue wet weight.

2.4. Radioligand binding study

To characterize the muscarinic receptors in the swine myometrium, we carried out a receptor binding assay using a nonselective muscarinic receptor radioligand, [^{3}H]quinuclidinyl benzilate. The myometrial membrane of the swine uterus was prepared by methods described previously (Taneike et al., 1991; Kitazawa et al., 1997). The circular muscle preparation was cut into small pieces and homogenized in 10 volumes of ice-cold Tris-buffer solution (50 mM Tris, 10 mM $MgCl_2$, pH = 7.4 at 4°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 $400 \times g$ for 10 min at 4°C, and the pellet was discarded. The supernatant was centrifuged at 40,000 $\times g$ for 30 min. The resulting pellet was washed twice. The washed pellet was suspended in 2.0 ml of the incubation buffer and used as a crude membrane preparation for determination of [^{3}H]quinuclidinyl benzilate binding. Protein in the membrane preparation was measured according to the method of Lowry et al. (1951).

The membrane preparation (200–400 μg protein/tube) was incubated with six increasing concentrations of the

radioligand (0.077–3.15 nM) in 200 μl of Tris-buffer (at 25°C for 60 min). The binding reaction was stopped by adding ice-cold buffer (4 ml), and the mixture was then filtered through a glass-fiber filter (GF/C; Whatman) under vacuum. The filter was then rapidly washed 6 times with 3 ml of ice-cold incubation buffer and placed in 20-ml glass scintillation vials with 0.5 ml of distilled water and 8 ml of scintillation fluid (Scintisol, EX-H, Dojin). Radioactivity trapped on the filter paper was counted in a liquid scintillation spectrometer (LCS-700; Aloka). Specific binding was calculated by subtracting nonspecific binding from total binding. Nonspecific binding was determined in the presence of 10 μM atropine. The maximum number of binding sites/mg of extract protein (B_{max}, concentration of receptors) and the equilibrium dissociation binding constant (K_d) were estimated by Scatchard analysis. Lines of fit were calculated by linear regression analysis using the least-squares method.

The muscarinic receptor subtype in the swine myometrium was characterized on the basis of the rank order of potency of five muscarinic receptor antagonists (atropine, pirenzepine, AF-DX116, 4-DAMP, tropicamide) for displacing specific [^{3}H]quinuclidinyl benzilate (1 nM) binding. The competition curves from four to five independent experiments for each antagonist were analyzed by a nonlinear, least-squares regression analysis of the binding data fitted to the Hill equation, which determines the IC_{50} values and slopes (Hill coefficients, nH). The dissociation constant (K_i) was calculated from the IC_{50} value of each antagonist by the method of Cheng and Prusoff (1973): $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of the radioligand (1 nM).

2.5. Chemicals

The following chemicals were used in the present experiments: acetylcholine chloride (Wako), atropine sulfate (Wako), carbachol chloride (Wako), clonidine hydrochloride (Sigma), 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide, RBI), forskolin (Wako), himbacine (Sigma), McN-A-343 (4-(*m*-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium chloride, RBI), methacholine chloride (Wako), muscarine hydrochloride (Wako), oxotremorine methiodide (oxotremorine-M, RBI), pirenzepine dihydrochloride monohydrate (Wako), pilocarpine hydrochloride (Tokyo Kasei), and tropicamide (Sigma). AF-DX116 (11-[[2-[(diethylamino)methyl] -1-piperiinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3*b*][1,4]benzodiazein-6-one) was kindly donated by Boehringer Ingelheim Drugs except for tropicamide, AF-DX116 and forskolin were dissolved in distilled water and applied directly to the organ bath. Tropicamide and forskolin were dissolved in ethanol, and the solution was diluted in distilled water and Krebs solution. AF-DX116 was dissolved in 1 N HCl and then diluted in distilled water and Krebs solution. The maximum concentration of ethanol in the bathing solution was set below 0.2% and that of HCl was also set below 0.001 N. These concentrations did not change the spontaneous contractile activity of the swine myometrium in the present experiments.

2.6. Statistical analysis

The results of the experiments are expressed as means ± S.E.M. of more than four individual experiments. Statistical analysis was performed by using paired and unpaired-*t* tests, with $P < 0.05$ as the criterion of statistical significance.

3. Results

3.1. Contractile response to muscarinic receptor agonists

Acetylcholine (1 nM–100 μM) applied to the organ bath caused concentration-dependent contractions with a $-\log EC_{50}$ value of 6.12 ± 0.13 ($n = 6$). The contractile responses were reproducible, as evidenced by the concentration–response curves recorded at 45-min intervals. $-\text{Log}EC_{50}$ and the maximum response to acetylcholine (first curve = 100%) were 6.27 ± 0.15 and 105 ± 5.3% (second curve), 6.25 ± 0.1 and 104 ± 4.5% (third curve), 6.23 ± 0.16 and 108 ± 5.6% (fourth curve), and 6.29 ± 0.11 and 108 ± 3.2% (fifth curve), respectively ($n = 6$). The contraction elicited by acetylcholine was not decreased by tetrodotoxin (1 μM), indicating the direct action of acetylcholine on uterine smooth muscle cells. Oxotremorine-M ($-\log EC_{50} = 6.47 \pm 0.04$, $n = 4$), methacholine (6.35 ± 0.15, $n = 4$), carbachol (6.18 ± 0.08, $n = 4$) and muscarine (6.33 ± 0.21, $n = 4$) also caused contractions, with a similar maximum amplitude. The relative amplitude (% of 100 μM acetylcholine-induced contraction) of the maximum response was 105 ± 4.1% ($n = 4$) for oxotremorine-M, 95 ± 3.2% ($n = 4$) for methacholine, 97 ± 2.5% ($n = 4$) for carbachol, and 94 ± 4.5% ($n = 4$) for muscarine. The present results suggested that these agents act as full agonists in the swine myometrium. Pilocarpine caused only weak contractions even at a high concentration (100 μM, 12 ± 2.8% of the 100 μM acetylcholine-induced response, $n = 13$). McN-A-343 was almost ineffective in causing mechanical responses in the myometrial circular muscles (Fig. 1).

The effects of pilocarpine or McN-A-343 on the contractile response to acetylcholine were investigated. Both agents inhibited acetylcholine-induced contractions and caused a rightward parallel shift of the concentration–response curve (data not shown). The $-\log EC_{50}$ value and amplitude of the maximum contraction in the presence of 100 μM pilocarpine and 100 μM McN-A-343 were 3.92 ± 0.05 and 91.2 ± 4.1% ($n = 5$), and 4.74 ± 0.04 and 80 ± 7.9% ($n = 5$), respectively, indicating that the in-

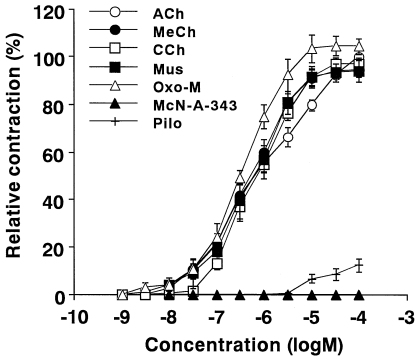

Fig. 1. Concentration–response curves of some muscarinic receptor agonists in the circular muscle of the swine myometrium. Each symbol shows the contractile response to acetylcholine (ACh, ○), methacholine (MeCh, ●), carbachol (CCh, □), muscarine (Mus, ■), oxotremorine-M (Oxo-M, △), McN-A-343 (▲) and pilocarpine (Pilo, +). Ordinate scale: relative amplitude of contraction expressed as a percentage of the 100 μM acetylcholine-induced contraction. Abscissa scale: concentration of muscarinic receptor agonists (log *M*). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

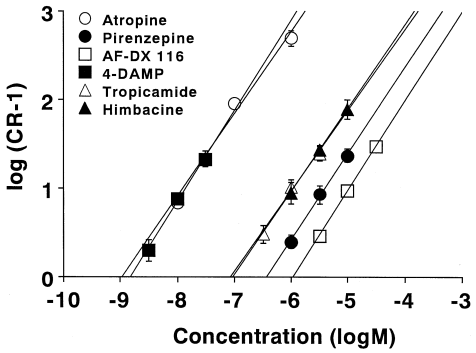

Fig. 2. The antagonism by six muscarinic receptor antagonists of the acetylcholine-induced contraction in the circular muscle of the swine myometrium. The inhibitory effects of atropine (○), pirenzepine (●), AF-DX116 (□), 4-DAMP (■), tropicamide (△) and himbacine (▲) on the concentration–response curves of acetylcholine were evaluated as the log of the EC_{50} ratio (CR) − 1 (ordinate) according to the procedure of Arunlakshana and Schild (1959) [see Section 2]. Abscissa scale: concentration of antagonists (log *M*). Each point represents the mean of four to seven individual experiments.

hibitory response of pilocarpine was about 10 times stronger than that of McN-A-343.

3.2. Characterization of acetylcholine-induced contractions by muscarinic receptor antagonists

Since the contraction elicited by acetylcholine was reproducible at 45-min intervals, we examined the effects of muscarinic receptor antagonists on the response to acetylcholine in the same preparations and the receptor subtype mediating the contraction was identified. First, we confirmed that atropine inhibited acetylcholine-induced contractions. Three increasing concentrations of atropine (10 nM, 100 nM, 1 μM) shifted the concentration–response curve of acetylcholine in a parallel rightward direction without affecting the maximum contraction (data not shown). The $-\log EC_{50}$ values of acetylcholine in the presence of atropine were 5.41 ± 0.12 (10 nM, $n = 5$), 4.3 ± 0.1(100 nM, $n = 5$) and 3.46 ± 0.05 (1 M, $n = 5$), respectively. The Schild plot of the inhibitory effect of atropine was linear ($r = 0.83$), and its slope (0.94 ± 0.06, $n = 5$) was not significantly different from unity. The pA_2 value of atropine for the acetylcholine-induced response was estimated to be 8.95 ± 0.05 ($n = 5$) (Fig. 2, Table 1).

Next, we examined the effects of five muscarinic receptor antagonists on acetylcholine-induced contractions. Like atropine, pirenzepine (1, 3, 10 μM), AF-DX116 (3, 10, 30 μM), 4-DAMP (3, 10, 30 nM), himbacine (1, 3, 10 μM) and tropicamide (300 nM, 1, 3 μM) antagonized the contractions evoked by acetylcholine in a concentration-dependent manner, and they caused a parallel shift of the concentration–response curve to the right without decreasing the maximum contraction. However, none of these antagonists changed the myometrial tonus, frequency or amplitude of the spontaneous contractions when they were applied to the organ bath within the concentration range used in this study. The Schild plot of the inhibitory effect of each antagonist on the response to acetylcholine was linear (correlation of regression line, $r = 0.8$–0.96). The pA_2 value and Schild slope of the antagonists were 6.42 and 0.98 for pirenzepine, 5.96 and 1.01 for AF-DX116, 8.83 and 1.02 for 4-DAMP, 7.07 and 0.92 for tropicamide, and 7.01 and 0.94 for himbacine, respectively. The Schild slope for each antagonist was not significantly different from unity (Fig. 2 and Table 1). The rank order of the antagonist potency was 4-DAMP > tropicamide ≥ himbacine > pirenzepine > AF-DX116.

3.3. Effects of muscarinic receptor antagonists on electrical field stimulation-induced contractions

Electrical field stimulation (10 Hz for 60 s) at 6-min intervals caused a reproducible contractile response which

Table 1
Potency of six muscarinic receptor antagonists for antagonizing acetylcholine-induced contraction in the circular muscle of the swine myometrium

Antagonists	pA_2	Slope	r
Atropine	8.95 ± 0.05	0.94 ± 0.06	0.83
Pirenzepine	6.42 ± 0.05	0.98 ± 0.11	0.84
AF-DX116	5.96 ± 0.03	1.01 ± 0.06	0.96
4-DAMP	8.83 ± 0.05	1.02 ± 0.13	0.82
Tropicamide	7.07 ± 0.05	0.92 ± 0.11	0.8
Himbacine	7.01 ± 0.07	0.94 ± 0.13	0.83

Values are means ± S.E.M. of four to seven individual studies. The antagonist action of each receptor antagonist was estimated by Schild plot analysis (see Fig. 2), and pA_2 values and the slope of the linear regression were calculated. Correlation coefficient (r) of the regression line is also indicated in the table. The slope of the plot for each antagonist was not significantly different from unity (1.00).

was abolished by tetrodotoxin (1 μM). As previously reported (Taneike et al., 1991, 1994), the field stimulation-induced contraction in the circular muscle was also inhibited by atropine in a concentration-dependent manner. These results suggested the involvement of cholinergic neurons and muscarinic receptors in the field stimulation-induced contraction. Next, the effects of five muscarinic receptor antagonists on the electrical field stimulation-induced cholinergic contraction were examined. As indicated in Fig. 3, pirenzepine (10 nM–10 μM), AF-DX116 (10 nM–10 μM), 4-DAMP (0.1 nM–100 nM), himbacine (10 nM–10 μM) and tropicamide (10 nM–10 μM) concentration dependently inhibited the contractile response to electrical field stimulation. The rank order of inhibition ($-\log IC_{50}$) was atropine (8.63 ± 0.6, $n = 4$) > 4-DAMP (8.35 ± 0.21, $n = 5$) > tropicamide (6.72 ± 0.13, $n = 4$) > himbacine (6.54 ± 0.35, $n = 4$) > pirenzepine (6.31 ± 0.11, $n = 4$) > AF-DX116 (6.13 ± 0.48, $n = 4$).

3.4. Effect of acetylcholine on cyclic AMP production

Fig. 4A shows the effects of acetylcholine (3 μM) on the tissue cyclic AMP level and on myometrial smooth muscle tension. The contractile response to acetylcholine developed quickly, was sustained for about 30 s, and then faded gradually. The relative amplitude of the contraction after a 5-min incubation with acetylcholine (3 μM) was $55 \pm 2.6\%$ ($n = 5$) of the maximal contraction. However, the tissue cyclic AMP level (control: 309 ± 91 pmol/g tissue wet weight, $n = 5$) did not significantly change during the contractile response in the swine myometrium. Tissue cyclic AMP levels for 1-, 2-, 5-min stimulation with 3 M acetylcholine were 295 ± 56 ($n = 4$), 275 ± 19 ($n = 4$), and 298 ± 43 pmol/g tissue wet weight ($n = 5$), respectively.

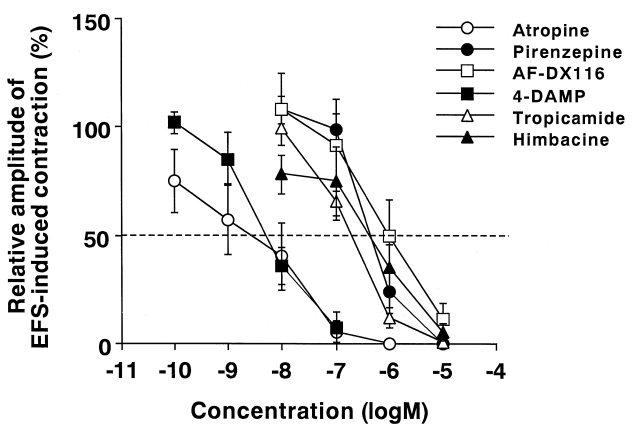

Fig. 3. Inhibition of electrical field stimulation-induced contractions by muscarinic receptor antagonists in the circular muscle of the swine myometrium. Each symbol shows the effect of atropine (○), pirenzepine (●), AF-DX116 (□), 4-DAMP (■), tropicamide (△) and himbacine (▲) on the contractions induced by electrical field stimulation (0.5 ms duration, 10 Hz for 60 s at 6-min intervals). Ordinate scale: electrical field stimulation-induced contraction in the absence of the antagonists was taken as 100%. Abscissa scale: concentration of antagonists ($\log M$). Points represent the means of four or more experiments with S.E.M. shown by vertical lines.

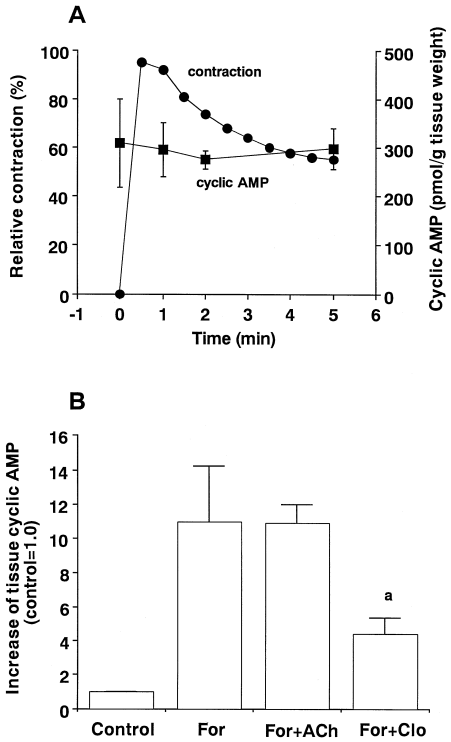

Fig. 4. Effect of acetylcholine on cyclic AMP production in the circular muscle of the swine myometrium. (A) Time course of acetylcholine (3 M)-induced contraction (from 0 to 5 min, ●) and tissue cyclic AMP level (■) during the contractile response (0, 1, 2, 5 min). Ordinate left: relative contraction is expressed as a percentage of the maximum amplitude of the acetylcholine (3 μM)-induced contraction, right: tissue cyclic AMP level (pmol/g tissue wet weight). Abscissa: time after application of acetylcholine (min). (B) Effect of acetylcholine and clonidine on the tissue cyclic AMP level elevated by forskolin. Each column represents the relative cyclic AMP level in the absence (control) and presence of forskolin (3 μM for 10 min), forskolin + acetylcholine (10 μM for 5 min) (For + ACh) and forskolin + clonidine (10 μM for 5 min) (For + Clo). Ordinate: relative increase of tissue cyclic AMP level from the control (1.0). Points and columns represent the means of four or more experiments with S.E.M. shown by vertical lines. (a) Significantly different from forskolin.

In the next experiment, the effect of acetylcholine on the tissue cyclic AMP level elevated by forskolin was investigated. Pretreatment with 3 μM forskolin for 10 min caused a 11 ± 3.2-fold ($n = 7$) increase in the tissue cyclic AMP level, but this stimulatory effect of forskolin was not significantly affected by a relatively high concentration of acetylcholine (10 μM) for 5 min (10.9 ± 1.1, $n = 8$). An α_2-adrenoceptor agonist, clonidine (10 μM), significantly decreased the tissue cyclic AMP level elevated by forskolin (4.35μ1.0, $n = 6$) (Fig. 4B).

3.5. [^{3}H]Quinuclidinyl benzilate binding study

For further characterization of the muscarinic receptor in the swine myometrium, the concentration of

[^{3}H]quinuclidinyl benzilate binding sites in the myometrial membrane and displacement of the binding by muscarinic receptor antagonists were examined. Binding of the radioligand to crude membrane preparations from the swine myometrium was a saturable process. Specific binding increased as the free concentration of the ligand increased (0.077–3.15 nM) and reached a plateau (Fig. 5A). Scatchard analysis of saturation binding parameters revealed the presence of a single class of binding sites (correlation of regression line, $r = 0.95–0.99$). From the regression line, K_d and B_{max} values were estimated to be 0.92 ± 0.03 nM ($n = 4$) and 126.6 ± 0.95 fmol/mg protein ($n = 4$), respectively (Fig. 5B). Specific binding for the radioligand (0.6 nM) at a concentration close to the K_d value indicated 97% of total binding. Hill plots of the binding data were linear with a Hill coefficient of 1.05 ± 0.04 ($n = 4$), which was not significantly different from unity (1.00), indicating that there was no positive or negative cooperativity in binding profiles.

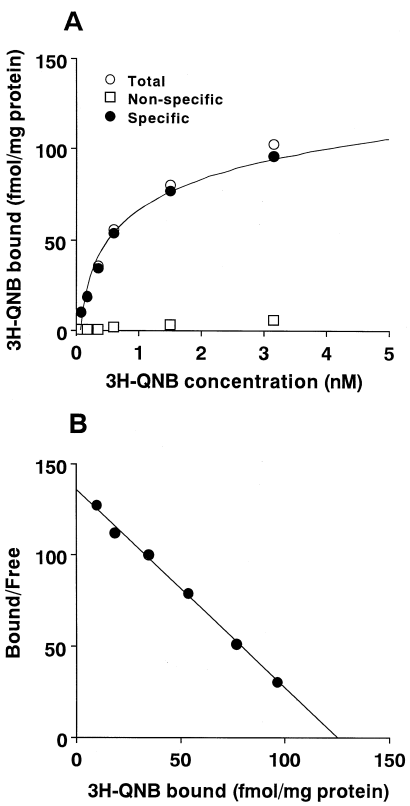

Fig. 5. [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB) binding to swine uterine circular muscle. (A) Crude membrane preparations obtained from circular muscles were incubated with increasing concentrations of [^{3}H]QNB (0.077–3.15 nM) for 60 min at 25°C in the absence (total binding, ○) and presence of 10 μM atropine (nonspecific binding, □). Specific binding (●) was determined as the difference between total and nonspecific bindings. Abscissa: [^{3}H]QNB concentration (nM). Ordinate: [^{3}H]QNB bound (fmol/mg protein). (B) Scatchard plot of the binding data in the circular muscle. The slope was determined by linear regression analysis. The points shown are from one of four or five similar experiments.

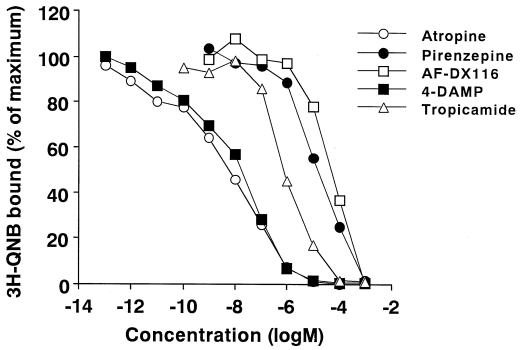

Fig. 6. Displacement of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding by muscarinic receptor antagonists. Competition curves for atropine (○), pirenzepine (●), AF-DX116 (□), 4-DAMP (■) and tropicamine (△) on [^{3}H]QNB (1 nM) binding to the circular muscle membrane of the swine myometrium Ordinate scale: [^{3}H]QNB binding (dpm) in the absence of antagonists was considered as 100%. Abscissa scale: concentration of antagonists ($\log M$). The points shown are from one of four or five similar experiments.

Five muscarinic receptor antagonists (atropine, pirenzepine, AF-DX 116, 4-DAMP, tropicamide) were examined for their influence on specific [^{3}H]quinuclidinyl benzilate (1 nM) binding. As indicated in Fig. 6, each antagonist concentration dependently inhibited the specific binding in a monophasic manner and eventually completely displaced it. The potency (K_i) in competing for the specific binding and Hill coefficient was 8.22 ± 0.16 and 0.93 ± 0.09 for atropine ($n = 4$), 8.18 ± 0.19 and 0.94 ± 0.05 for 4-DAMP ($n = 4$), 6.78 ± 0.09 and 0.93 ± 0.07 for tropicamide ($n = 5$), 5.46 ± 0.09 and 0.92 ± 0.07 for pirenzepine ($n = 4$), 5.12 ± 0.07 and 0.94 ± 0.05 for AF-DX 116 ($n = 5$), respectively. Hill coefficients for each antagonist were not significantly different from unity (1.00). These results supported the notion of the presence of a single class of receptor and no cooperativity between binding sites.

The correlation between pA_2 and pK_i values of five muscarinic receptor antagonists (atropine, pirenzepine, AF-DX 116, 4-DAMP and tropicamide) was 0.98 (significant, $P < 0.05$), and the slope of the regression line was 0.93, which was not significantly different from 1.0.

4. Discussion

Many studies with gastrointestinal (rat ileum, guinea-pig ileum, dog colon), tracheal (cow, dog, rat) and urinary (rat, pig) smooth muscles have demonstrated the coexistence of muscarinic M_2 and M_3 receptors. Evidence for this has been obtained from competitive radioligand binding studies and immunoprecipitation of muscarinic receptors (Levey, 1993; Eglen et al., 1996; Ehlert et al., 1997a). However, in the smooth muscle tissues of some animals, it is the coupling of the muscarinic M_3 receptor to a phosphoinositide-specific phospholipase C that mediates the

major part of the contractions induced by muscarinic receptor agonists. The role of the muscarinic M_2 receptor (inhibition of adenylate cyclase activity) in mediating the smooth muscle contraction has not been clarified. However, under experimental conditions where intracellular cyclic AMP is elevated by forskolin or isoproterenol, muscarinic M_2 receptor stimulation can elicit indirect contraction of smooth muscle by inhibiting the relaxant effect of forskolin or isoproterenol on histamine-induced contractions. The mechanism for this indirect contraction involves the muscarinic M_2 receptor-mediated inhibition of cyclic AMP accumulation (Thomas and Ehlert, 1996; Ehlert et al., 1997b). In the case of uterine smooth muscle, the muscarinic M_2 receptor has been shown to be the dominant receptor subtype in rats (Pennefather et al., 1994) and in guinea-pigs (Doods et al., 1993), and to mediate the contraction of muscarinic receptor agonists (Eglen et al., 1989). Additionally, recent studies also suggested the involvement of muscarinic M_3 and M_4 receptors in the contraction of the guinea-pig uterus by muscarinic receptor agonists (Dörje et al., 1990; Leiber et al., 1990). In the present experiment, the binding study (saturation and displacement studies) strongly suggested the presence of a single class of binding site for [^{3}H]quinuclidinyl benzilate (muscarinic receptor) in the swine myometrial membrane. Furthermore, we have shown that the muscarinic receptor present in the swine uterus is of the M_3 subtype and plays a functional role in the contractions induced by both endogenous and exogenous acetylcholine. The conclusion that the muscarinic M_3 receptor mediates the contractile response to acetylcholine is based on the inhibitory actions of atropine and five muscarinic receptor antagonists (pirenzepine, AF-DX116, 4-DAMP, himbacine and tropicamide) that possess varying degrees of affinity for different muscarinic receptor subtypes (M_1, M_2, M_3 and M_4) (Hulme et al., 1990; Dörje et al., 1991; Doods et al., 1993; Eglen et al., 1996; Ehlert et al., 1997b). In the present pharmacological attempt to define the receptor subtype, all the antagonistic agents used had the properties of competitive antagonists, shifting the concentration–response curve of acetylcholine to the right in a parallel manner, but without reducing the maximum amplitude of the acetylcholine induced contraction.

The antagonist profile of pirenzepine at various muscarinic receptor subtypes is $M_1 > M_4 > M_3 > M_2$ (Doods et al., 1993; Eglen et al., 1996), which is a similar to the profile for the human cloned muscarinic receptor (Dörje et al., 1991). In our study, the pA_2 value of pirenzepine (6.42) approached that observed for the M_3 receptor in the guinea-pig ileum (6.5–7.2), guinea-pig bladder (6.6–6.8), and human colon (6.87, 7.23) (Eltze et al., 1993; Kerr et al., 1995; Eglen et al., 1996), but this value of pirenzepine was about 100 times lower than that for muscarinic M_1 receptors (8.08, 8.3, Eltze et al., 1993; Eglen et al., 1996). This suggests that activation of the muscarinic M_1 receptor does not contribute to the contraction evoked by acetylcholine in the swine myometrium. McN-A-343 ($m_1 = m_4 > m_3 > m_2$) and pilocarpine ($m_1 = m_3 > m_2 = m_4$) show relative selectivity toward the M_1 subtype (Eltze et al., 1993; Schwarz et al., 1993). Both of these agonists were very weak contractile agents in the swine myometrium and this result indirectly supports the conclusion of the antagonist study. However, it appears that the weak efficacy of pilocarpine and McN-A-343 to produce contraction does not reflect their inability to access the muscarinic M_3 receptor, because the acetylcholine-induced contraction was antagonized by both agents in a competitive manner. The antagonist-like action of pilocarpine has been already demonstrated in the guinea-pig myometrium (Leiber et al., 1990).

AF-DX116 is a muscarinic M_2 receptor-selective antagonist and showed a potency profile of $M_2 > M_1 \geq M_4 > M_3$ in a functional study (Giachetti et al., 1986) and a similar profile for the human cloned muscarinic receptor (Dörje et al., 1991). The pA_2 value of AF-DX116 for muscarinic receptors in the swine myometrium (5.96) was conspiciously lower than that obtained in the guinea-pig atrium (7.33, 7.71), which expresses mainly muscarinic M_2 receptors, but it was close to that found in the guinea-pig ileum (6.44, 6.63), guinea-pig trachea (6.24) and guinea-pig urinary bladder (6.24), where muscarinic M_3 receptors mediate the contraction elicited by muscarinic receptor agonists (Giachetti et al., 1986; Dorofeeva et al., 1992). Himbacine is another muscarinic M_2 receptor antagonist that has a similar antgonist potency for the muscarinic M_4 receptor ($M_2 \geq M_4 > M_1 > M_3$, Doods et al., 1993; Lazerano and Birdsall, 1993; Eglen et al., 1996). The pK_b values of himbacine for muscarinic M_2 (8.29) and M_4 (8.13) receptors (Lazerano and Birdsall, 1993) are considerably higher than the pA_2 value of himbacine in the swine myometrium (7.01), and this value is almost identical with the pK_b value for the M_3 subtype (7.04, Lazerano and Birdsall, 1993). These results exclude the possible involvement of the muscarinic M_2 receptor subtype in the contractile response to acetylcholine.

The anatagonist profile of tropicamide is $M_4 > M_3 \geq M_2 > M_1$ and this antagonist is used to characterize muscarinic M_4 receptors, because a useful M_4 receptor anatgonist is not available at present. The antagonist activities (pK_b, pK_i) of tropicamide toward muscarinic M_3 and M_4 receptors are 7.18–7.34 (M_3) and 7.54–8.13 (M_4), respectively (Doods et al., 1993; Lazerano and Birdsall, 1993; Dei et al., 1996). In the present study, the pA_2 value of tropicamide (7.07) against the acetylcholine-induced contraction was more similar to that for its activity for the M_3 receptor than that for the M_4 receptor. In addition, himbacine had a lower potency (pA_2, 7.01) for swine uterine muscarinic receptors than for muscarinic M_4 receptors (8.13) (Lazerano and Birdsall, 1993). These results indicate that activation of muscarinic M_4 receptors does not contribute to the contractions elicited by acetylcholine in the swine myometrium.

The antagonist profile for 4-DAMP is $M_3 > M_1 \geq M_4 > M_2$ (Eglen et al., 1996) with a similar profile for human cloned muscarinic receptors (Dörje et al., 1991; Hegde et al., 1997) and this agent is currently used to characterize muscarinic M_3 receptor-mediated responses. The pA_2 value of 4-DAMP (8.83) against the acetylcholine-induced contraction in the present study was almost consistent with the antagonist potency obtained in the guinea-pig ileum (8.83, Eltze et al., 1993), human colon (9.09, Kerr et al., 1995) and mouse airways (8.9, Garssen et al., 1993), which express the muscarinic M_3 receptors involved in the contractile response. The similarity of the pA_2 values of muscarinic receptor antagonists (pirenzepine, AF-DX116, himbacine, tropicamide, 4-DAMP) toward the M_3 subtype indicates the involvement of muscarinic M_3 receptors in the contractile response to acetylcholine in the swine myometrium.

Comparison of the rank order of muscarinic receptor antagonist potency is another way to identify the receptor subtype present in a tissue. The rank order of potency of the antagonists used in the present study differs according to the muscarinic receptor subtype. For example, 4-DAMP > pirenzepine > tropicaminehimbacine > AF-DX116 at the M_1 type; 4-DAMP > himbacine > AF-DX116 tropicamine > pirenzepine at the M_2 type; 4-DAMP > tropicamide > himbacine > pirenzepine > AF-DX116 at the M_3 type; and 4-DAMP > himbacine > tropicamide > pirenzepine > AF-DX116 at the M_4 type (Lazerano and Birdsall, 1993; Doods et al., 1993; Eglen et al., 1996; Hegde et al., 1997). The order of the antagonist potency (pA_2) in the swine myometrium was 4-DAMP > tropicamide ≥ himbacine > pirenzepine > AF-DX116, and this order is consistent with that obtained for the displacement of [^{3}H]quinuclidinyl benzilate binding (pK_i; 4-DAMP > tropicamide > pirenzepine > AF-DX116) and there was a significant correlation between them. The rank order of antagonists in the swine myometrium was quite different from that of muscarinic M_1 and M_2 receptors but similar to that of muscarinic M_3 or M_4 receptor subtypes. Although the rank order of the muscarinic receptor antagonist potency was close, muscarinic M_3 and M_4 receptors can be discriminated by the difference in the pK_i value between 4-DAMP and pirenzepine (pK_i of 4-DAMP − pK_i of pirenzepine), because the difference is 2.2–2.5 for M_3 and 1.4–1.5 for M_4 type (Dörje et al., 1991; Doods et al., 1993; Eglen et al., 1996). Differences in pA_2 and pK_i values between these two antagonists were 2.41 (pA_2) and 2.72 (pK_i) in the present study. These findings support the conclusion that the muscarinic M_3 receptor mediates the contractile response to acetylcholine in the swine myometrium.

m_5 is another type of muscarinic receptor whose functional role has not yet been clarified. The rank order of pK_i for this muscarinic receptor subtype is 4-DAMP (8.98) > pirenzepine (7.05) > himbacine (6.31) (Dörje et al., 1991). The rank order of these three antagonists and the pK_i values of pirenzepine and himbacine were quite different from those obtained in the swine myometrium, indicating that it is unlikely that the muscarinic receptor mediating the contractile response to acetylcholine is of the m_5 subtype.

In the radioligand displacement study, although Hill coefficients were not significantly different from unity, the pK_i values for the antagonists were slightly lower than the pA_2 values obtained in the contraction study. At present, we cannot clearly explain the reasons for the different values but dissociation between the two values has been already indicated in functional and binding studies with muscarinic receptor antagonists (Eglen et al., 1996). Because the pK_i value reflects the interaction between antagonist and receptor only and the pA_2 value reflects the interaction between antagonist and receptor-linked contractile mechanisms, a slight difference might be observed between them.

Electrical field stimulation-induced contractions in the circular muscle of the swine uterus were completely inhibited by tetrodotoxin and atropine, indicating that endogenous acetylcholine released from cholinergic nerves acts on muscular muscarinic receptors and causes contraction, as previously demonstrated (Taneike et al., 1991, 1994). Muscarinic receptor antagonists also decreased the field stimulation-induced contraction in a concentration-dependent manner. The rank order of inhibition (4-DAMP > tropicamide > himbacine > pirenzepine > AF-DX116) was consistent with that of exogenous acetylcholine (pA_2). The similar rank order of the muscarinic receptor antagonists suggests that the M_3 subtype mediates the contraction induced by not only bath applied (exogenous) acetylcholine but also endogenous acetylcholine released by electrical field stimulation.

Muscarinic M_2 and M_4 receptors are linked to the inhibition of adenylate cyclase activity, and activation of these receptor subtypes lowers tissue cyclic AMP levels (Eglen et al., 1996; Ehlert et al., 1997a). To investigate the possible involvement of muscarinic M_2 and/or M_4 receptors in the contractile response to acetylcholine, the effect of acetylcholine on cyclic AMP production was investigated. In the swine myometrium, the tissue cyclic AMP level was not significantly changed by acetylcholine, regardless of the presence or absence of forskolin (under conditions of an increased tissue cyclic AMP level). However, stimulation of α_2-adrenoceptors (negatively coupled to adenylate cyclase, Ruffolo et al., 1991) by clonidine significantly decreased the forskolin-induced increase in tissue cyclic AMP level under these experimental conditions. The failure of acetylcholine to decrease the cyclic AMP level suggested the absence of functional muscarinic M_2 and/or M_4 receptors coupled to inhibition of adenylate cyclase activity and is consistent with the results of the present study.

In summary, the swine uterus contains a single muscarinic receptor (M_3 subtype), and both exogenous and

endogenous acetylcholine cause contraction of the myometrium through activation of this receptor subtype present on smooth muscle cells. This conclusion is drawn on the basis of the results of pharmacological analysis of the acetylcholine-induced contraction and of [^{3}H]quinuclidinyl benzilate binding using six different muscarinic receptor antagonists (atropine, pirenzepine, AF-DX116, 4-DAMP, himbacine and tropicamide).

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