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MUSCARINIC RECEPTORS IN ISOLATED SMOOTH MUSCLE CELLS FROM GASTRIC ANTRUM

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Abstract—Smooth muscle cells from the gastric antrum of the rabbit were isolated using collagenase and pronase. We examined the characteristics of muscarinic receptors that control contraction of the muscle cell: kinetics, stoichiometry and specificity of both contractile response to muscarinic agents and binding of labeled *N*-methyl-scopolamine. Cells contracted in the presence of muscarinic agents after a short time (30 sec) while binding of (^{3}H)-NMS reached a plateau after 10 min exposure. Specific binding was saturable and Scatchard analysis revealed a single class of high-affinity binding sites (K_d: 0.5 nM). Oxotremorine was the most potent agonist with an ED_{50} of 0.6 pM; acetylcholine and carbachol were 10 times less potent. Muscarinic antagonists competed with (^{3}H)-NMS for binding with IC_{50} values in the same range (nanomolar or less) than those obtained for inhibition of acetylcholine-induced contractions. Pirenzepine antagonized contractile effect of muscarinic agonists with EC_{50} in a micromolar range. Intracellular levels of cyclic AMP were lowered by muscarinic agonists. Monoclonal anti-muscarinic receptor antibodies M-35 displayed agonist-like activities triggering contraction and lowering cyclic AMP levels of the cells. However, although the antagonist inhibits M-35-induced contractions and cAMP decrease, M-35 had no effect on binding of the antagonist to the muscarinic receptor. These data revealed the presence of an M_2-muscarinic receptor subtype involved in the contractile response of the isolated smooth muscle cell.

Muscarinic receptors are present both in the mucosa and in smooth muscles of the stomach wall [1]. Contractions induced by cholinergic drugs in connection with binding of muscarinic antagonists, *N*-methyl-scopolamine or quinuclidyl-benzilate, have been demonstrated in isolated smooth muscle cells from amphibian [2], guinea-pig [3, 4], canine [5] and human [6] stomachs. Isolated smooth muscle cells, as well as isolated muscle strips, are good tools for measuring contraction and binding on the same *in vitro* preparation, but it has been demonstrated that isolated smooth muscle cells were markedly more sensitive than muscle strips to the contractile effect of a large variety of agents [5]. On smooth muscle cells isolated from canine gastric corpus, Collins *et al.* [5, 7, 8] demonstrated, from NMS binding data, the presence of muscarinic receptors with high affinity for antagonists and low affinity for agonists suggesting the existence of muscarinic receptor heterogeneity. In addition, they showed that these receptors were of M_2-subtype, i.e. with low affinity for pirenzepine. The same observation came from our previous studies on isolated secretory cells from rabbit fundus where acetylcholine-induced acid secretion was also mediated through a muscarinic receptor M_2-subtype (K_d for pirenzepine: 0.1 μM) coupled with intracellular changes in inositol-trisphosphate [9, 10]. Monoclonal antibodies to purified muscarinic receptor were recently prepared [11]. One of them, called M-35, was shown to mimic muscarinic agonist in the guinea-pig myometrium [12]: just like carbachol, M-35 induces uterine contraction and causes a rise in the intracellular cyclic GMP levels together with a concomitant decrease of cyclic AMP accumulation induced by prostacyclin, indicating that this anti-receptor antibody was able to mimic all the neurotransmitter-induced contractions and confirming that this information is entirely contained in the receptor.

In this paper, our purpose was threefold: first, to study the relationships between the characteristics of the muscarinic receptor (analysed by labeled antagonist binding) and those coming from contractile responses to muscarinic agents on a dispersed cell preparation from antral rabbit smooth muscles; second, to examine the effects of muscarinic agents on cyclic AMP cellular levels; third, to study the effects of the M-35 monoclonal antibody on both muscarinic receptor and cyclic AMP cellular levels. These investigations would give us more information on the cholinergic control of the functions of the stomach.

MATERIALS AND METHODS

Collagenase from *Clostridium histolyticum* was from Serva (F.R.G.). Pronase was from Boehringer-Mannheim (F.R.G.). Soybean trypsin inhibitor (STI), bovine serum albumin (BSA) fraction V, scopolamine, atropine, quinuclidyl benzilate (QNB), oxotremorine, pilocarpine, carbamoylcholine, acetylcholine, 3-isobutylmethylxanthine (IBMX) were from Sigma Chemical Co. (St Louis, MO). Pirenzepine was a gift from Dr. Hammer (Boehringer-Ingelheim, F.R.G.). Earle's balanced salt solution was from Bio-Merieux (France). (^{3}H)-*N*-methyl-sco-

polamine (NMS) (80 Ci/mmol), cyclic AMP RIA kit and scintillation liquid (ACS) were from Amersham (U.K.).

Monoclonal anti-muscarinic receptor M-35 antibody was prepared as previously [11]. Protein concentrations of the IgM solution (determined with Bradford's method) was 1.5 mg/ml.

Medium A: 132 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM HEPES, 0.2% glucose, 0.2% BSA, 0.02% phenol red, pH 7.4.

Medium B: Earle's balanced salt solution containing 10 mM HEPES and 0.2% BSA, pH 7.4.

PBS: phosphate buffered saline solution, pH 7.4.

Isolation of smooth muscle cells from gastric antrum of rabbit. Smooth muscle cells from the gastric antrum of a rabbit were prepared by a modification of the method previously described by Bitar *et al.* [6]. After removing the stomach, the antral part was extensively washed with iced PBS solution. The serosal and mucosal layers were peeled off the muscular layers. Muscle tissues were minced into small pieces of 2–3 mm^2 and incubated in medium A containing 0.25% collagenase, 0.04% pronase, 0.01% STI and gassed with 100% O_2. After 60 min at 30°, the incubation medium was filtered through a nylon mesh. The filtrate which contained isolated cells was diluted with medium A and centrifuged at 150 *g* for 5 min. The cell pellet was then diluted in medium B. The remaining tissue from the first incubation was reincubated in fresh medium A for 30 min at 30°.

Tissue fragments were dispersed into single cells by passages in and out of the inverted wide end of a 5 ml pipette. The resultant cell suspension was filtered through a nylon mesh. The isolated cells from the two incubations were pooled and counted. Viability (estimated by trypan blue exclusion) was always greater than 90%. This protocol usually gives about 1×10^7 cells per stomach.

Measurements of contraction of isolated cells. To 0.1 ml of medium B containing the agents to be tested, 0.5 ml of the cell suspension (6×10^4 cells) was added. The temperature was maintained at 30°. After 30 sec, the reaction was stopped by addition of 0.1 ml glutaraldehyde solution to get a final glutaraldehyde concentration of 2%. In control experiments, 0.1 ml medium B was used instead of the agent solution. The mean cell length in the presence or not of the contractile agents was statistically determined by video–microscopic measurements (NIKON microscope with a JVC video camera) of about 100 cells. The contractile response to an agonist was expressed as the percentage of decrease of the averaged cell length compared to the mean length of the control cells.

Binding studies. In standard binding assays, aliquots of the cell suspension containing about 160,000 cells were incubated in small tubes with (^{3}H)-*N*-methyl-scopolamine [(^{3}H)-NMS] (6.5×10^{-10} M) in the presence or not of competitors in Earle's solution (0.2 ml final volume) for 20 min at 30°. In saturation studies, various concentrations of (^{3}H)-NMS from 0.4 to 20 nM were incubated with the same amount of isolated cells in the same experimental conditions. Separation of bound from free fractions was achieved by addition of 0.6 ml of ice-cold Earle's solution and immediate centrifuge at 10,000 *g* for 1 min in a microfuge. The cell pellet was resuspended in 0.1 ml 10% $HClO_4$ and put into vials containing 10 ml ACS for the determination of radioactivity with a liquid scintillation counter. Non-specific binding was determined by measurements of bound radioactivity in the presence of an excess (1×10^{-6} M) of unlabeled scopolamine.

Cyclic-AMP determinations. Cyclic AMP content of isolated smooth muscle cells was measured by radioimmunoassay as follows: the cell suspension (150,000 cells per ml) was preincubated for 5 min in Earle's solution at 30° and 0.4 ml of the suspension was incubated in duplicate with various concentrations of muscarinic agents or M-35 antibody in the presence of 1×10^{-5} M IBMX for 5 min at 30°. Cells were centrifuged and resuspended in 0.4 ml Earle's solution. Then 0.1 ml 40% trichloracetic acid was added. Cyclic AMP was measured in duplicate on 0.1 ml neutralized aliquots by radioimmunoassay. The amount of cyclic AMP was expressed as picomoles per 150,000 cells.

RESULTS

Figure 1 illustrates typical isolated smooth muscle cells obtained with this technique. The averaged resting length (Fig. 2) measured on a series of 108 cells was 135 ± 5 μm with a range of 70–220 μm.

Agents inducing contraction of isolated cells

Incubation of cells with various concentrations (1×10^{-14} M to 1×10^{-5} M) of muscarinic agonists carbachol, acetylcholine, oxotremorine, resulted in a dose-dependent decrease in cell length (Fig. 3). The lowest concentration required for a significant response was less than 1×10^{-13} M with these three agents. The maximum decrease in cell length was about 40% and occurred for 1×10^{-7} M. The ED_{50} values were respectively 5.6×10^{-13} M for oxotremorine, 7.3×10^{-12} M for acetylcholine and 9.5×10^{-12} M for carbachol. Oxotremorine was 10 times as potent as acetylcholine or carbachol.

Effect of muscarinic antagonists on contractions induced by acetylcholine

The muscarinic antagonists (atropine, QNB, scopolamine and pirenzepine) decreased the sensitivity of the smooth muscle cells to acetylcholine. The maximal response induced by 1×10^{-7} M acetylcholine was inhibited by these agents and the parallelism of the curves shown on Fig. 4 confirmed the competitive character of this inhibition. These data also revealed high sensitivity for QNB and low sensitivity for pirenzepine. IC_{50} values were respectively 2×10^{-12} M for QNB, 5×10^{-10} M for atropine, 2×10^{-9} M for scopolamine and 6×10^{-6} M for pirenzepine.

(^{3}H)-N-methyl-scopolamine binding studies

Specific binding of labeled *N*-methyl-scopolamine was linearly related ($r = 0.997$) to the number of cells in the range from 7,500 to 225,000 cells per ml (Fig. 5). Specific NMS binding reached a plateau after 20 min incubation at 30° and this steady state remains stable up to 60 min (Fig. 6).

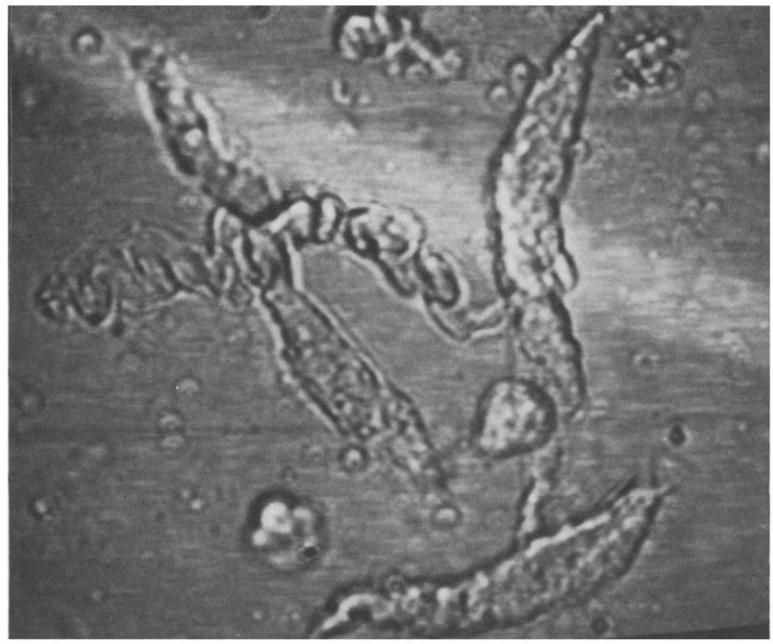

Fig. 1. Photomicrography of isolated cells from the circular smooth muscle of rabbit gastric antrum. The video-microscopic observation of the preparation was carried out after fixation with glutaraldehyde as described in Materials and Methods (original magnification ×200).

When reducing incubation temperature, the time required to reach equilibrium dramatically increased: the binding was time- and temperature-dependent. Specific (^{3}H)-NMS binding was a saturable function of the ligand concentration (Fig. 7). Moreover, non-specific binding was unsaturable and increased linearly with tracer concentration. Scatchard's plot was linear (linear regression $r = 0.992$) in agreement with the presence of a single class of high-affinity binding sites. The K_d value was

$$5 \pm 1.6 \times 10^{-10}\,\text{M}\ (\text{N} = 6)$$

and the number of sites was about 100,000 per cell.

Specific (^{3}H)-NMS binding was inhibited in a dose-dependent manner by the agents believed to act at the cholinergic muscarinic receptor (Fig. 8). The IC_{50} values were listed in Table 1. The antagonists QNB, atropine and scopolamine had IC_{50} in the nanomolar range, while muscarinic agonists oxotremorine, acetylcholine, carbachol and pilocarpine had IC_{50} in the micromolar range.

Pirenzepine also competitively inhibited (^{3}H)-NMS binding with an IC_{50} of about 6×10^{-6} M (20,000 times higher than scopolamine). Hill plots

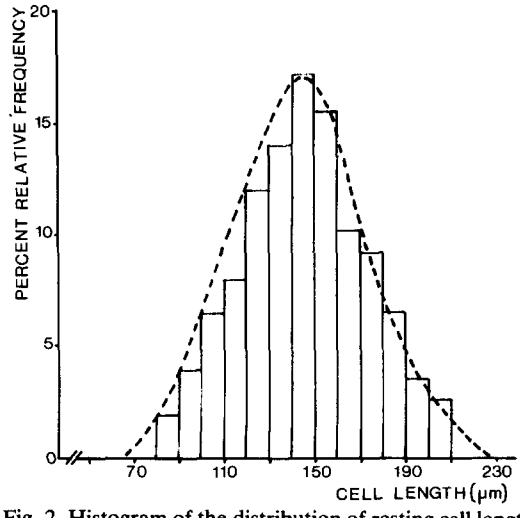

Fig. 2. Histogram of the distribution of resting cell lengths. Cells were isolated and fixed with glutaraldehyde for video-microscopic measurement as described under Materials and Methods.

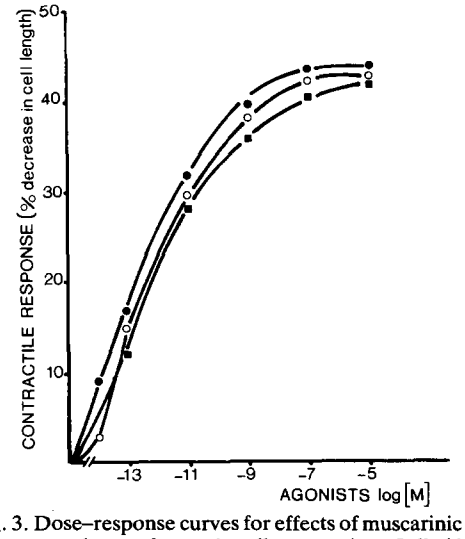

Fig. 3. Dose–response curves for effects of muscarinic agonists on antral smooth muscle cell contraction. Cells (6×10^4 cells in 0.5 ml) were incubated in standard incubation buffer with indicated concentrations of agonists for 30 sec at 30°. After fixation, the cell length of about 100 cells was determined as described under Materials and Methods. Each value is the mean of three separate experiments. (■): Carbachol, (○): Acetylcholine, (●): Oxotremorine.

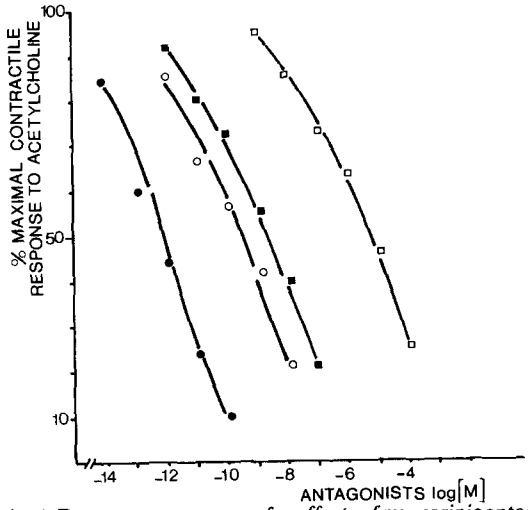

Fig. 4. Dose–response curves for effects of muscarinic antagonists on cell contraction induced by acetylcholine. Cells (6×10^6 cells in 0.5 ml) were incubated with acetylcholine (1×10^{-7} M) and indicated concentrations of antagonists (QNB, atropine, scopolamine, pirenzepine) as described under Materials and Methods. After fixation, the cell length of about 100 cells was determined as described under Materials and Methods. Results were expressed as the percent of the maximal contractile response to 1×10^{-7} M acetylcholine. Each value is the mean of three separate experiments. (●): QNB, (○): atropine, (■): scopolamine, (□): pirenzepine.

from these data led to Hill coefficients near to 1 for antagonists and near to 0.5 for agonists, in agreement with those observed for muscarinic receptors from other tissues (gastric secretory mucosa, heart, brain).

Cyclic AMP contents of isolated smooth muscle cells

Incubation of cells with carbachol resulted in a dose-dependent decrease in cAMP contents of the isolated smooth muscle cells (Fig. 9). Resting levels of cAMP were 87.5 ± 7.5 pmol/150,000 cells (5 experiments) and the minimal value after carbachol was 10 ± 4.1 pmol/150,000 cells (88% decrease). Both atropine (1×10^{-8} M) and pirenzepine (1×10^{-5} M) prevented this inhibition (Table 2).

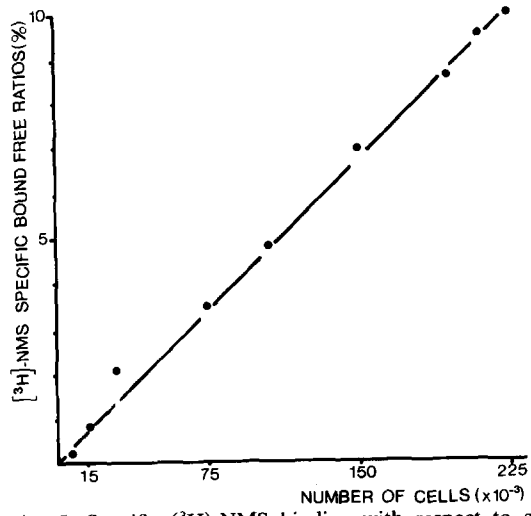

Fig. 5. Specific (^{3}H)-NMS binding with respect to cell number. Cells at indicated number were incubated with (^{3}H)-NMS (6.5×10^{-10} M) for 20 min at 30°. Bound radioactivity was determined as described under Materials and Methods. Specific bound to free ratios were plotted against the number of cells. Results are means from 3 separate experiments.

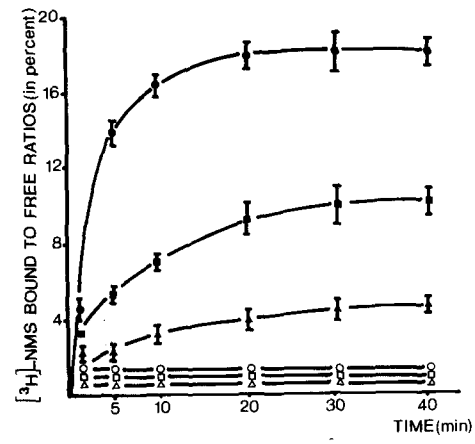

Fig. 6. Time-course of (^{3}H)-NMS binding on isolated smooth muscle cells. (^{3}H)-NMS (6.5×10^{-10} M) was incubated with cells (160,000 cells) at different temperature in the absence (total binding) or in the presence (non-specific binding) of 1 μM scopolamine. Mean results from 3 separate experiments at indicated temperatures expressed as bound to free ratio were plotted as a function of time. Vertical error bars indicated standard deviations of 3 separate experiments. Total binding: (●): 30°, (■): 20°, (▲): 4°. Non-specific binding: (○): 30°, (□): 20°, (△): 4°.

Effect of M-35 monoclonal antibody on isolated smooth muscle cells

Incubation of cells with various concentrations of M-35 antibody resulted in a dose-dependent decrease

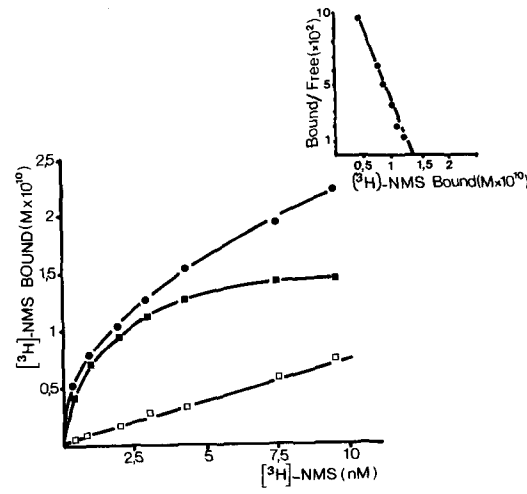

Fig. 7. Association of (^{3}H)-NMS with smooth muscle cells at steady state. Various concentrations of (^{3}H)-NMS (0.4–20 nM) with (□) or without (●) scopolamine (1 μM) were incubated with cells for 20 min at 30°. Binding was expressed as bound labeled ligand as a function of (^{3}H)-NMS concentrations. Specific binding (■) is the difference between total and non-specific binding. Values are means from six separate experiments. Insert: Scatchard's plot from these data yielded a K_d of 5×10^{-10} M and a B_{max} of 1.3×10^{-10} M.

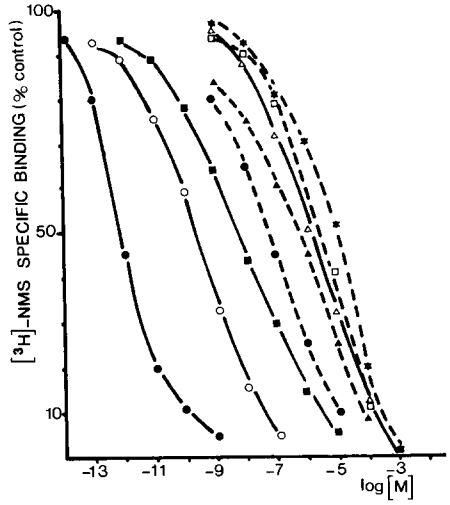

Fig. 8. Dose–response curves for effects of muscarinic agonists and antagonists on (^{3}H)-NMS binding to smooth muscle cells. (^{3}H)-NMS (6.5×10^{-10} M) was incubated with cells for 20 min at 30° in the presence of varying concentrations of unlabeled compounds. Results were expressed as the percent of maximal specific binding and plotted against concentrations of the respective compounds. Results are means of 3–6 separate experiments. (●) QNB, (○) scopolamine, (■) atropine, (△) pirenzepine, (□) pilocarpine, (●) oxotremorine, (★) carbachol, (▲) acetylcholine.

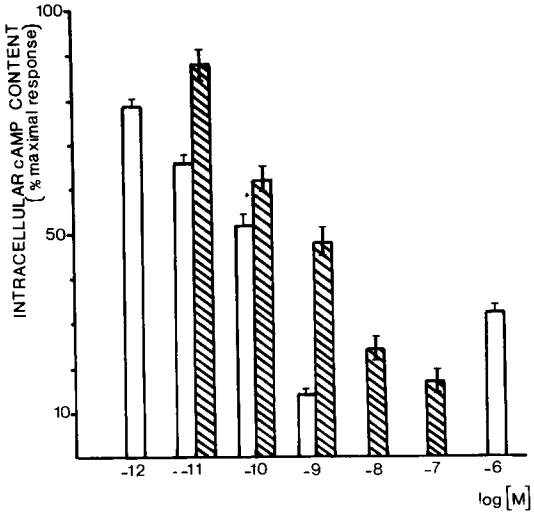

Fig. 9. Cyclic AMP contents of isolated smooth muscle cells in the presence of carbachol or M-35 antibody. Cells were incubated with indicated concentrations of carbachol or dilutions of M-35 antibody for 5 min at 30° in the presence of 1×10^{-5} M IBMX. Cyclic AMP levels were determined by radioimmunoassay in duplicate. Results are expressed as % of the maximal cAMP content determined on control cells (without carbachol or M-35). Errors bars indicated standard deviations from 3 separate experiments. □, carbachol; ▨, M-35 antibody.

in cell length (Fig. 10). The maximal decrease in cell length was 43% for the higher antibody concentration tested. The muscarinic antagonist atropine, even at high concentration (1×10^{-7} M), failed to prevent M-35-induced contractions.

M-35 antibody played a similar role to carbachol on cyclic AMP accumulation (Fig. 9): the maximal decrease was 83% with the undiluted antibody and this decrease in cyclic AMP levels was prevented by atropine or pirenzepine, but to a lesser extent (Table 2). This inhibition of cyclic AMP contents of isolated smooth muscle cells from gastric antrum was in the same range as that observed on prostacyclin-stimulated guinea-pig myometrium with the same agents. However, this antibody was unable, even at high concentration, to inhibit the specific binding of (^{3}H)-NMS to muscarinic receptors of this cell preparation.

DISCUSSION

Contraction and relaxation of smooth muscle circular fibers from gastric antrum are under both cholinergic and peptidergic controls. Previous works have shown that muscarinic receptors mediated the local action of acetylcholine on the isolated antral smooth muscle cells from various species.

The results shown in this paper confirm the possibility of isolating functional cells from the circular antral smooth muscle of rabbit and providing evidence for the presence of muscarinic cholinergic receptors mediating contraction in this isolated cell preparation. The mean resting cell length and its reduction in the presence of a maximal acetylcholine concentration were similar to those previously described in other tissues [3–5]. The order of sen-

Table 1. IC_{50} values (and Hill coefficients (nH)) for the inhibition of (^{3}H)-NMS binding by muscarinic agonists and antagonists to isolated smooth muscle cells

Antagonists	IC_{50} (M)	Agonists	IC_{50} (M)
QNB	$2.1 \pm 0.8 \times 10^{-12}$	Oxotremorine	$7.1 \pm 2.5 \times 10^{-8}$
nH	0.97	nH	0.56
Atropine	$5.0 \pm 2.1 \times 10^{-9}$	Acetylcholine	$6.0 \pm 1.5 \times 10^{-7}$
nH	0.95	nH	0.40
Scopolamine	$3.0 \pm 1.5 \times 10^{-10}$	Pilocarpine	$1.2 \pm 0.6 \times 10^{-6}$
nH	1.03	nH	0.40
Pirenzepine	$6.1 \pm 2.4 \times 10^{-6}$	Carbachol	$9.1 \pm 1.1 \times 10^{-6}$
nH	0.83	nH	0.60

Results are means (±SD) from 3 separate experiments.

(^{3}H)-NMS binding assay. So, we can suppose that M-35 antibody interacts with the muscarinic receptor through a distinct proteic domain than does muscarinic agonists, without modification of the second messenger mediation of this contractile effect.

In conclusion, single cells obtained from the antral gastric smooth muscle contain efficient muscarinic receptor sites of M_2-subtype capable of triggering the contraction of the cells. The use of the monoclonal anti-receptor antibody confirms that the transmission of the signal to the muscle cell is due to information entirely contained in the muscarinic receptor.

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