Human esophageal smooth muscle cells express muscarinic receptor subtypes M₁ through M₅

JIAN WANG,1–3 PAWEL S. KRYSIAK,2,3 LISANNE G. LAURIER,1,3 STEPHEN M. SIMS,2 AND HAROLD G. PREIKSAITIS1–3

Departments of 1Medicine and 2Physiology, The University of Western Ontario, and 3Lawson Research Institute, St. Joseph’s Health Centre, London, Ontario, Canada N6A 4V2

Received 21 December 1999; accepted in final form 31 May 2000

Acetylcholine is the major excitatory neurotransmitter in the gastrointestinal tract. Atropine inhibits the motor effects of cholinergic nerves on the gastrointestinal tract, indicating the importance of muscarinic cholinergic receptors (11, 12). Five subtypes of the muscarinic ACh receptor (mAChR), encoded by five distinct genes, have been identified (M₁–M₅) (4, 5, 24). M₁ receptors have been localized to the myenteric plexus, where they function as presynaptic autoreceptors to enhance ACh release from nerves (19). Receptor-ligand binding studies (11, 12) have demonstrated the presence of M₂ and M₅ receptors on gastrointestinal smooth muscle. Although the M₂ receptor subtype is the most abundant, in many preparations contraction is mediated primarily by the M₃ receptor (11, 12). However, some studies also support a role for M₅ or M₁ receptors in contraction of cat esophageal (30) and guinea pig gallbladder smooth muscles (23). Recently (37), it has been shown that the M₂ receptor activates a nonselective cation channel in guinea pig terminal ileum but requires concurrent M₃ receptor stimulation to do so, thus complicating the interpretation of antagonist effects. The resulting depolarization would activate L-type Ca²⁺ channels and contribute to muscle contraction. These observations indicate that the distribution and function of receptor subtypes involved in gastrointestinal smooth muscle function may be more complex than originally envisioned.

Receptor-ligand binding and muscle contraction studies that rely on pharmacological approaches for mAChR identification are limited by the poor subtype selectivity of agonists and antagonists. Using more sensitive immunologic techniques, Wall et al. (33) showed the presence of M₁, M₂, and M₅ receptors in guinea pig ileum and Dorje et al. (10) have shown M₁, M₂, M₃, and M₄ receptors in rabbit ileum. mRNA encoding M₁, M₂, M₃, and M₄ has been demonstrated by RT-PCR in human gallbladder smooth muscle (18). To date, a possible role for the M₅ receptor in gastrointestinal smooth muscle has received relatively little attention, although the M₅ receptor has now been identified in several peripheral, nonneuronal tissues (1, 6, 13, 14, 22, 31).

As has been pointed out, there is a relative paucity of information regarding the expression and function of mAChRs in the human esophagus and other gastrointestinal smooth muscles (11, 12). Evidence from both in vitro and in vivo studies (9, 32) supports an essential...
role of mAChR activation in esophageal peristalsis. The recent report (16) of anti-M2 autoantibodies in patients with Chagas disease of the esophagus, a condition in which esophageal peristalsis is impaired, highlights the potential clinical importance of mAChR subtypes in the pathogenesis of esophageal motor disorders. However, the expression or function of the receptor subtypes in the human esophagus has not been adequately examined. Using human esophageal smooth muscle, we (29) previously demonstrated that cholinergic excitation involves multiple sources of Ca\(^{2+}\), including release from intracellular stores and influx through voltage-dependent L-type Ca\(^{2+}\) channels. This suggests the possible involvement of several mAChR subtypes working through different signaling pathways. For example, M2 receptor activation of a nonselective cation current would allow influx of Ca\(^{2+}\) through L-type Ca\(^{2+}\) channels (34, 37), whereas M1, M3, or M5 receptor activation of the inositol 1,4,5-trisphosphate (IP\(_3\)) pathway would result in the release of Ca\(^{2+}\) from intracellular stores (11, 12).

The roles of various mAChR subtypes in the control of esophageal peristalsis in animal models is also not completely resolved. 4-Diphenylacetoxyl-N-methyl piperidine methiodide (4-DAMP; an M1/M5-selective antagonist) was shown to inhibit peristalsis in the cat (2) and opossum (15), whereas pirenzepine (an M2-selective antagonist) had little effect. These findings support a major role for the M3 receptor subtype in mediating esophageal peristalsis, similar to contraction of other gastrointestinal smooth muscles (11, 12). On the other hand, ACh-induced shortening of acutely dispersed myocytes from the cat esophagus was shown to be mediated by the M2 receptor subtype (30). Although we (27) previously demonstrated that subtype-selective antagonists displayed a pattern more consistent with the M3 receptor in blocking contraction of cat esophageal smooth muscle strips, a possible contribution by other subtypes cannot be ruled out.

In the present study, we used a combination of molecular and immunologic techniques to examine the expression of mAChR subtypes in human esophageal smooth muscle cells (SMCs) in primary culture. We were surprised to find evidence for the presence of all five known mAChR subtypes in these SMCs. We demonstrate functional cholinergic responses using fura 2 Ca\(^{2+}\) fluorescence and compare the mechanisms of cholinergic excitation in cultured cells to those we (29) have described previously in freshly dispersed cells and muscle strips. We anticipate that this cell culture system will provide a useful model for studying esophageal smooth muscle physiology and receptor signaling mechanisms.

**METHODS AND MATERIALS**

**Tissue retrieval and SMC culture.** Tissue collection was performed in accordance with the guidelines of the University of Western Ontario Review Board for Research Involving Human Subjects. Tissues were obtained from a disease-free region of the midportion of the distal third of the esophagus obtained from patients undergoing esophagectomy for cancer. The longitudinal muscle (LM) and circular muscle (CM) layers were separated and carefully cleaned of nerves, blood vessels, fat, and fascia. SMCs were dispersed by incubating with 1.7 mg/ml collagenase, 0.5 mg/ml elastase, and 1 mg/ml BSA in Hanks’ balanced salt solution (HBSS; GIBCO BRL) for 45 min at 37°C. The dispersed cells were washed in HBSS and seeded onto 10-cm culture plastic plates at a density of ~400 cells/cm\(^2\) in DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cells were grown in a humidified atmosphere of 5% CO\(_2\) in air at 37°C. Cells were examined and photographed using an Olympus IMT-2 inverted microscope using phase-contrast optics. Only primary cultures were used in the present study.

**Immunocytochemistry.** For fluorescence immunocytochemistry (ICC) SMCs were grown as described above on sterile 13-mm glass coverslips placed into culture dishes. Cells were fixed in ice-cold 95% ethanol (for 15–30 min), blocked with 4% goat serum in PBS (20 min), and incubated with primary antibody at 4°C. Primary antibodies were visualized with anti-mouse or anti-rabbit secondary antibodies (1:100, Jackson Immunoresearch Laboratories, West Grove, PA). TO-PRO-1 dimeric cyanine dye (5 μl/ml, 1 h at 22°C, Molecular Probes, Eugene, OR) was used to stain nuclei. Coverslips were mounted on slides with FluoroGuard Antifade (Bio-Rad, Hercules, CA). For negative controls, primary antibodies were omitted or preadsorbed with the appropriate peptide immunogen and processed as described above. Peptide immunogens were obtained from Research and Diagnostic Antibodies and corresponded to the following amino acid sequences: M1 receptor, 451–460; M2 receptor, 457–466; M3 receptor, 580–589; M4 receptor, 469–478; and M5 receptor, 519–531.

To assess purity, cultured SMCs were incubated with primary antibodies directed against a variety of markers: rabbit polyclonal anti-S100 (1:6,400, Innovation Foundation, Toronto, ON, Canada), mouse monoclonal anti-CD34 (1:50, Novo Castra Laboratories, Newcastle upon Tyne, UK), and rabbit polyclonal anti-c-Kit (1:200, Santa Cruz Biotechnology). The protocol described above was used with the following modifications: blocking with normal rabbit serum (20 min at 22°C), incubating with primary antibodies overnight at 4°C, and using the standard avidin-biotin-peroxide complex-3,3′-diaminobenzidine technique for detection according to the manufacturer’s suggested protocol (Vector Laboratories, Burlingame, CA). Sections were counterstained with Carazzi’s hematoxylin. A Zeiss Axioskop fluorescence microscope was used to examine cells, with images captured with a Sony DXC-950 3CCD color video camera and processed using Northern Eclipse image analysis software (Empix Imaging, Mississauga, ON, Canada).

**RNA isolation and RT-PCR.** Total RNA was extracted from SMCs grown to near confluence (days 10–14) by acid guanidinium thiocyanate-phenol-chloroform extraction. The quality of the RNA was verified by agarose gel electrophore-
sis using ethidium bromide staining. Four micrograms of total RNA were reverse-transcribed with random hexamers using a first-strand cDNA synthesis kit (Pharmacia Biotech, Madison, WI). Three microliters of the cDNA reaction mixture were used in each PCR reaction. PCR was performed in a 50-μl reaction containing PCR buffer, 2 mM MgCl₂, 200 μM dNTPs, 0.1 nM of each primer, and 2 units of Taq DNA polymerase (Qiagen, Valencia, CA). PCR was carried out in a GeneAmp 2400 PCR thermal cycler (Perkin-Elmer, Norwalk, CT) for 28–33 cycles. Cycling parameters were 94°C for 1 min, 52–60°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR oligonucleotide primers used to amplify cDNA are listed in Table 1. PCR primers for β-actin were used to confirm fidelity of the PCR reaction and to detect genomic DNA contamination. The amplified products (10 μl) were analyzed by electrophoresis on 1% agarose-TAE [10 mM Tris (pH 7.5), 5.7% glacial acetic acid, and 1 mM EDTA] gels and visualized by ethidium bromide staining. Sequencing of PCR products for verification was done in the Robarts Research Institute Core Molecular Biology Facility (London, ON, Canada).

**Immunoblotting.** SMCs grown to or near confluence were scraped from the culture plates and washed in PBS. The cell pellet was homogenized (Brinkmann Polytron) in 10 mM HEPES buffer (pH 7.0) containing 1 mM dithiothreitol and Mini Complete protease inhibitor cocktail tablets (1 tablet/25 ml buffer, Boehringer Mannheim, Indianapolis, IN). Crude protein homogenates were denatured by boiling for 15 min, separated on 10% polyacrylamide gels (10 ml/lane), and electrophoretically transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham, Piscataway, NJ) in buffer containing 25 mM Tris, 250 mM glycine, and 20% methanol (pH 8.3). Membranes were blocked overnight at 4°C with 4% goat serum and 30% goat’s glycine, and 20% methanol (pH 8.3). Membranes were incubated with primary anti-mAChR antibodies and processed as described above.

**Measurement of intracellular Ca²⁺.** Free intracellular Ca²⁺ concentration ([Ca²⁺]i) was determined in SMCs grown on 13-mm coverslips using fura 2. Cells were loaded by incubation with 0.2 μM fura 2-AM for 20–40 min at 29°C. The fura 2-loaded cells on the coverslips were placed in a 0.75-ml chamber mounted on a Nikon inverted microscope and superfused at 2–5 ml/min at room temperature. Cells were illuminated with alternating 345- and 380-nm light using a Deltascan system (Photon Technology International, London, ON, Canada), with emission detected by a photomultiplier at 510 nm. After correction for background fluorescence, [Ca²⁺]i was calculated as previously described (29) using a viscosity factor of 15% and a dissociation constant of 225 nM for binding of Ca²⁺ to fura 2. Test agents were applied by bath perfusion or by pressure injection from glass micropipettes positioned ~50 μm from the cell. Control applications of vehicle had no effect on [Ca²⁺]i. Muscarinic receptor subtype antagonists were applied at concentrations indicated for at least 1 min before rechallenge with ACh. The fractional block of each mAChR antagonist was predicted as described by Wang et al. (34) using the formula: %bound = 1/(1 + [D/Kd]), where D is the concentration of each antagonist and Kd is the published equilibrium dissociation constant of each antagonist for each receptor subtype (11, 12). The superfusion buffer had the following composition (in mM): 130 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 20 HEPES, and 10 d-glucose, adjusted to pH 7.4. Ca²⁺-free buffer also contained 0.5 mM EGTA to chelate residual Ca²⁺.

**Drugs and materials.** Unless stated otherwise, all chemicals were obtained from Sigma Chemical (St. Louis, MO) or BDH (Toronto, ON, Canada). Fura 2-AM (Molecular Probes) was prepared in DMSO. Nifedipine, BAY K8644, cyclopiazonic acid (CPA), methoctramine, pirenzepine, and 4-DAMP were obtained from Research Biochemicals International (Natick, MA). All drugs were prepared as concentrated stock solutions and diluted into the appropriate bathing solution before addition to cells.

**Statistics.** The composite data are expressed as means ± SE. Statistical analyses were performed with a two-tailed paired Student’s t-test; n refers to the number of cells tested. Differences were considered to be significant when P < 0.05.

## RESULTS

**Human esophageal SMCs in culture.** Dispersed human esophageal cells placed in culture began to show visible signs of growth after 2–3 days. Individual cells were phase bright and assumed a spindle-shaped appearance with cytoplasmic projections extending from a larger central area that contained the nucleus (Fig. 1). At confluence, human esophageal SMCs assumed a

### Table 1. PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primer Pair Sequence Sense/Antisense</th>
<th>Product Size, bp</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₁</td>
<td>X15263</td>
<td>5’-CAGGCAACCTGCTGTTACTC-3’/5’-CTGCTGCTGCTGTC-3’</td>
<td>538</td>
<td>222–759</td>
</tr>
<tr>
<td>M₂</td>
<td>X15264</td>
<td>5’-CTCTTCTCAAAATACCTGG-3’/5’-GGCTCCCTTGTGCTCTCT-3’</td>
<td>654</td>
<td>212–865</td>
</tr>
<tr>
<td>M₃</td>
<td>X15266</td>
<td>5’-GAAGGAGCAGAGGAGTGGCG-3’/5’-CTCGTCCTTACATGGTGG-3’</td>
<td>560</td>
<td>1,016–1,575</td>
</tr>
<tr>
<td>M₄</td>
<td>M16405</td>
<td>5’-ATGCTATGAGACGGTGCGG-3’/5’-GTTGACGAGATGGATAG-3’</td>
<td>503</td>
<td>174–677</td>
</tr>
<tr>
<td>M₅</td>
<td>M80333</td>
<td>5’-ACCACACCTCGCTGCTGCTC-3’/5’-CTCTTCCAGCTGCTCTC-3’</td>
<td>752</td>
<td>225–703</td>
</tr>
<tr>
<td>β-Actin</td>
<td>X00351</td>
<td>5’-CTCGTCATACTCCTGCTG-3’/5’-CTCGTCATACTCCTGCTG-3’</td>
<td>314</td>
<td>820–1,133</td>
</tr>
</tbody>
</table>

* β-Actin PCR primer pair was selected to span a 206-bp intron.
typical hill-and-valley appearance, (Fig. 1) as has been described for other SMCs in culture (8, 17). All nuclei were found to be contained in cells that stained positively with anti-smooth muscle α-actin antibody, indicating that cultures were of uniform cellular composition consisting of SMCs (Fig. 2). The α-actin immunostaining demonstrated structures organized into intracellular strands or bundles (Fig. 2), also typical of the appearance of α-actin in other types of SMCs in culture (8). Although virtually all SMCs stained positively for smooth muscle-specific α-actin, we tested for possible microscopic contamination by other cell types (Fig. 3) using primary antibodies directed against CD34 (endothelial cells), S100 (neuroglial and Schwann cells), and c-Kit (interstitial cells of Cajal). Only CD34 showed a small amount of staining (<4% as determined by cell counting; 4 esophageal specimens). In each case, appropriate positive controls were processed concurrently and showed typical immunostaining expected for each antibody tested (not shown), including human melanoma (S100), human spleen (CD34), and human colon, which contains interstitial cells of Cajal (c-Kit). Cells of CM or LM origin displayed similar growth characteristics, morphology, and immunostaining properties (Figs. 1 and 2).

RT-PCR identifies mRNA for all five muscarinic receptor subtypes. Ligand binding studies indicate that most SMCs express the M₂ mACHR subtype in abundance and lesser amounts of the M₃ mACHR (11, 12). One of the goals of this study was to identify which mACHRs are expressed in human esophageal smooth muscle. PCR primers were designed to amplify sequences specific for each of the five human mACHR subtypes. Transcripts for all five mACHRs were identified in cultured cells from both the LM and CM layers (Fig. 4). The identity of the amplified products was confirmed by direct DNA sequencing. The identification of a single RT-PCR product of predicted size for β-actin mRNA rules out contamination by genomic DNA, because the β-actin primers were designed to span a 206-bp intron.

Western blotting and ICC of muscarinic receptors. Protein expression of mACHR subtypes was determined by immunoblot analysis of homogenates of cultured esophageal SMCs. We used specific polyclonal antibodies directed at each of the five mACHR subtypes to examine their expression in LM and CM SMCs (Fig. 5). We identified immunoreactive protein bands corresponding to each receptor subtype and estimated their molecular weights to be as follows: M₁, 55; M₂ 52; M₃,
For each receptor antibody, a dominant single band was observed that was suppressed by preincubation of the primary antibodies with the respective peptide immunogen (not shown). Minor immunostaining bands observed with anti-M1, -M3, and -M4 were not similarly suppressed in the presence of immunogen peptide, and none of the minor bands corresponded to other receptor-related bands, indicating that they were not due to cross-reactivity of the antibodies. No protein bands were observed if primary antibody was omitted and immunoblots were incubated only with secondary anti-rabbit horseradish peroxidase-conjugated IgG antibody (Fig. 5).

Fluorescence ICC was used to examine the expression of mAChR subtypes in intact esophageal SMCs (Fig. 6). Positive immunostaining of all five mAChR subtypes was identified in SMCs with immunoreactivity to the mAChRs seen throughout the cells. Immunostaining was often associated with the plasma membrane and was seen also in the cytoplasm. No immunolabeling was observed if primary antibodies were preincubated with the appropriate immunogen peptide (not shown) or when primary antibodies were omitted and cells were incubated with secondary antibody only (Fig. 6). Similar findings were obtained for SMCs from LM and CM.

Cholinergic stimulation evokes increase in [Ca^{2+}]. To investigate cholinergic responses, [Ca^{2+}], was measured in fura 2-loaded cultured SMCs. Resting [Ca^{2+}] was not significantly different in LM and CM cells at 130 ± 20 and 135 ± 22 nM, respectively (n = 8, 3 specimens). Brief application of ACh (10 s) elicited a transient increase in [Ca^{2+}], of similar amplitude in LM and CM at 565 ± 30 and 543 ± 29 nM, respectively (n = 8, 3 specimens). As shown in Fig. 7, the response to ACh was preserved on removal of extracellular Ca^{2+} by bathing cells in Ca^{2+}-free medium containing 0.5 mM EGTA (Fig. 7, A and B). Subsequent responses to ACh gradually diminished, consistent with the slow depletion of intracellular Ca^{2+} stores, with some response still evident after >100 min in Ca^{2+}-free medium. The diminution of the response was not caused by cell run-down because substantial recovery was seen after return of Ca^{2+} to the bathing medium (Fig. 7A). Further evidence for the participation of stores...
was seen on the addition of CPA, which inhibits the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum. CPA, added in Ca\(^{2+}\)-free medium, rapidly and profoundly, but reversibly, inhibited the ACh-induced transient (Fig. 7B). These data support a major role for intracellular stores as the source for the ACh-induced rise in [Ca\(^{2+}\)]\(_i\). Despite the fact that ACh elicited large Ca\(^{2+}\) transients, accompanying contraction was not seen, in contrast to that seen for freshly dispersed cells (29). Similar results were obtained for cells cultured from LM or CM. We also examined the possible involvement of voltage-dependent L-type Ca\(^{2+}\) channels, because ACh, acting through M\(_2\) receptors coupled to a nonselective cation current, might contribute to membrane depolarization (34, 37). There was no significant effect of the L-type Ca\(^{2+}\) channel activator BAY K8644 or the L-type Ca\(^{2+}\) channel blocker nifedipine on the [Ca\(^{2+}\)]\(_i\) transient observed with brief application of ACh (10 s) in either LM or CM SMCs (Fig. 7C). As we (29) found for acutely isolated esophageal myocytes, prolonged application of ACh (30–300 s) produced a second plateau elevation of [Ca\(^{2+}\)]\(_i\), after the initial transient in 12 cells tested (Fig. 8). Moreover, in 11 additional cells, oscillations were also observed during the plateau. We considered the possibility that Ca\(^{2+}\) influx could contribute to this plateau phase, as previously demonstrated for acutely isolated myocytes (29). Removal of bathing Ca\(^{2+}\) (with 0.5 mM EGTA present) resulted in a prompt and reversible decline in the plateau [Ca\(^{2+}\)]\(_i\) (Fig. 8B, representative of 10 cells). A similar reduction of the plateau was observed on application of L-type Ca\(^{2+}\) channel blocker (3 cells, not shown). These observations indicate that Ca\(^{2+}\) influx contributes to the agonist-mediated response in esophageal SMCs in culture.

**Cholinergic response is mediated by M\(_3\) receptor.** Next, we sought to determine which mAChR subtype(s) mediated the response to ACh. It was not feasible to perform complete concentration-response analyses using fura 2 Ca\(^{2+}\) fluorescence, and highly selective agonists and antagonists that can discriminate among all five receptor subtypes are not available. Hence, we chose to focus on three antagonists that have been found to be most useful for functional differentiation of mAChRs. We examined the effects of 0.1 \(\mu\)M methoctramine (M\(_2/M_4\)-selective antagonist), 0.1 \(\mu\)M pirenzepine (M\(_1\)-selective antagonist), and 0.01 \(\mu\)M 4-DAMP (M\(_1/M_3\)-selective antagonist) on the ACh-induced [Ca\(^{2+}\)]\(_i\) transient (Fig. 9). The effects of the antagonists on the response to prolonged ACh application were not evaluated because the plateau elevation of [Ca\(^{2+}\)]\(_i\) was obscured by oscillatory activity as described above. These antagonists had similar effects on the ACh-induced [Ca\(^{2+}\)]\(_i\), transient in LM and CM SMCs. 4-DAMP almost abolished the response to ACh, reducing it to <4% of control in both CM and LM, whereas methoctramine suppressed the response to 79 ± 5% and 85 ± 2% of control in CM and LM, respectively. Pirenzepine had a slightly larger effect, suppressing the response to 65 ± 2% and 61 ± 3% of control in CM and LM, respectively. For all three antagonists, the ACh response recovered on washout of the antagonist. Using the known affinity constants of the receptor subtypes for these antagonists (11), we...
estimated that M₃ receptor sites should be blocked 93% by 0.01 μM 4-DAMP, close to that observed. The findings do not support a significant contribution by M₁, M₂, or M₄ receptors in this response. At the antagonist concentration used, pirenzepine would be expected to block 95% of M₁ and 83% of M₄ receptors, whereas methoctramine should block 89% of M₂ and 80% of M₄ receptors. Because M₃ and M₅ receptors have similar affinity profiles for these antagonists, a role for the M₅ receptor subtype cannot be excluded (see DISCUSSION).

**DISCUSSION**

We set out to examine the expression of mAChR subtypes in cultured SMCs from the human esophagus. Because previous studies (11, 12) identified mainly M₂ and M₃ receptors in a variety of gastrointestinal smooth muscles, we were surprised to find all five subtypes expressed in these cells. This finding is supported by RT-PCR demonstrating the presence of mRNA for the five receptors and immunoblotting and ICC demonstrating the presence of each receptor protein. The possible contribution of receptor mRNA or protein from other cell types is unlikely, based on the absence of significant contamination by neural elements, endothelial cells, or interstitial cells of Cajal, because specific markers for these cell types were absent or detected only in trace amounts.

The molecular weights we estimated based on immunoblot analysis for receptors M₁–M₄ are close to those predicted from the known amino acid sequences for each and also correspond well to their estimated sizes in other tissues (20–22). The band identified by the anti-M₅ antibody in our immunoblot analysis has a molecular mass of 80 kDa, which is significantly greater than that predicted from the known structure of the M₅ receptor protein (60 kDa), but less than the molecular mass estimated by Buchli et al. (6) and Ndoye et al. (22) based on immunoblotting (95 kDa). Muscarinic receptors have been reported (36) to form dimers and multimers through disulfide bond linkages. This is unlikely to account for the higher-molecular mass form of M₅ we identified, because a band of ~120 kDa would be expected on this basis and our experiments were done under reducing conditions that would disrupt disulfide bonds. We speculate that the discrepancy in predicted and observed molecular weight of the

![Figure 7](https://example.com/fig7.jpg)

**Fig. 7.** Cholinergic stimulation causes a transient rise of free intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in fura 2-loaded cultured SMCs. **A**: ACh (10 μM, applied for 10 s, arrowheads) transiently elevated [Ca²⁺]ᵢ, from resting levels. Control response (left) in 1 mM Ca²⁺-containing solution persisted on removal of extracellular Ca²⁺. With prolonged bathing in Ca²⁺-free solution, the ACh response was diminished, consistent with depletion of intracellular stores. At least a 10-min recovery of SMCs was allowed between successive stimulations, as indicated by breaks in the time record. Response recovered promptly on return of 1 mM Ca²⁺. **B**: cyclopiazonic acid (CPA, 10 μM), which blocks the sarcoplasmic reticulum Ca²⁺-ATPase, resulted in more rapid and profound attenuation of the ACh-induced [Ca²⁺]ᵢ, transient. Effect of CPA was reversible with recovery on washout and reintroduction of 1 mM Ca²⁺. **C**: influx of Ca²⁺ through L-type Ca²⁺ channels contributed little to response because neither channel opener BAY K8644 (BayK, 1 μM) nor channel blocker nifedipine (Nifed, 1 μM) had a significant effect on ACh-induced Ca²⁺ transient. Bar graph at right summarizes results for 10 cells derived from 3 specimens. Changes are not statistically significant (P > 0.10 for both nifedipine and BAY K8644 in both LM and CM cells). Representative traces are shown for cells cultured from CM. Similar results were obtained for cells from LM.
M₅ receptor might arise as a result of several mechanisms that have been reported for other G protein-coupled receptors (3), including posttranslational modification, genetic polymorphism, or the existence of splice variants, all of which could vary between tissues and cell types.

We are unaware of any other cell type that simultaneously expresses all five known mAChR subtypes.

Fig. 8. Prolonged application of ACh causes a transient rise of $[\text{Ca}^{2+}]_i$, followed by a plateau phase in fura 2-loaded cultured SMCs. 

A: ACh (10 μM, applied for 300 s) produced initial transient elevated $[\text{Ca}^{2+}]_i$, as with brief application (Fig. 7), followed by regular oscillations of $[\text{Ca}^{2+}]_i$ that in some cases persisted as long as ACh was applied. In the example shown, $[\text{Ca}^{2+}]_i$ oscillations settled to a steady plateau elevation of $[\text{Ca}^{2+}]_i$. B: in some cells, a plateau phase without oscillations was observed (left). Removal of extracellular $\text{Ca}^{2+}$ attenuated the plateau phase, indicating that $\text{Ca}^{2+}$ influx contributes (right). Examples shown are for cells cultured from the LM with similar findings observed for CM.

Fig. 9. Muscarinic antagonists display pattern of blockade consistent with the M₅ subtype. ACh (10 μM for 10 s, arrowheads) produced $[\text{Ca}^{2+}]_i$ transients blocked by mAChR antagonists. All antagonists produced significant blockade when applied for at least 1 min before rechallenge with ACh, but substantial differences in the magnitude of the effect are apparent. 4-Diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP, 0.01 μM; M₁/M₃ antagonist) profoundly blocked the response (A), whereas methoctramine (0.1 μM; M₂/M₄ antagonist) blocked 15–21% of the response (B) and pirenzepine (0.1 μM; M₁ antagonist) blocked 35–39% of the control response (C). At these antagonist concentrations, this blockade is anticipated based on the predicted receptor occupancy for the M₅ or M₆ subtypes (see RESULTS). Responses recovered on washout of antagonist (A–C). Representative traces for each antagonist effect on cells cultured from CM are shown. D: bar graph summarizes results for cells cultured from LM and CM. *P < 0.01, antagonist vs. control; n = 9–12 cells for each of LM and CM from 3–4 specimens.
Labeling of our SMCs by the five mACHR antibodies was ubiquitous. We identified some labeling in each cell in every field examined, suggesting that all SMCs expressed all receptor subtypes. This observation contrasts with findings of a recent study (17) in cultured airway smooth muscle, in which it was shown that M2 receptors were widely expressed, whereas M3 receptors were found predominantly only in certain cells that had undergone phenotypic change after serum withdrawal. Other mACHR subtypes were not examined in that study, but our finding of the presence of all five mACHRs in esophageal SMCs, maintained in serum-replete conditions, highlights a difference in the expression of at least the M3 receptor in these two SMC culture models. Recently, we (26) demonstrated the expression of all five mACHRs in SMCs acutely isolated from the human esophagus, indicating that the present observations are not due to a shift to a proliferative phenotype that occurs when SMCs are placed in culture (8).

The findings presented here do not support the commonly expressed view that gastrointestinal smooth muscles express only M2 and M3 receptors (11, 12), a conclusion based mainly on data derived from ligand binding experiments. The limitations of detecting more than two receptor subtypes, especially at low levels of expression, using a pharmacological approach are well recognized (12). Using more sensitive methods for receptor identification, Dorje et al. (10), Heinig et al. (18), and Wall et al. (33) found mACHR subtypes M1–M4 in gastrointestinal smooth muscles. It may be argued that the receptors detected in these studies originated not from smooth muscle but rather from other tissue elements such as enteric nerves, for which there are functional data demonstrating the presence of multiple mACHR subtypes including M1 and M4 (19). Our findings are particularly relevant in this regard, because we clearly demonstrated the expression of each mACHR subtype on the SMCs by ICC and our cultures were free of contamination by other cell types.

This study in human esophageal SMCs is the first to demonstrate that the M5 mACHR subtype is present in gastrointestinal smooth muscle. This tissue is added to the growing list of human nonneural tissues in which affinity labeling, molecular, and/or immunologic evidence shows the presence of this subtype, including skin fibroblasts and keratinocytes (6, 22), endothelial cells and smooth muscle of the neurovasculature (13), iris-ciliary body of the eye (14), and lymphocytes (1, 31). The M5 receptor was cloned from brain, but initial studies (5) failed to demonstrate significant mRNA for this receptor in all tissues tested, casting doubt on its physiological relevance. It was later shown by in situ hybridization and autoradiography that M5 has a specific distribution in brain that differs from the other subtypes, leading Reever et al. (28) to suggest a unique and biologically important role for this receptor. Recently (7), interest in the potential physiological role of M5 has been stimulated by the observation of unique structural features that permit the receptor to be constitutively activated in the absence of ligand. The importance of this to the multiple functions that are now attributed to various smooth muscle phenotypes remains to be determined (25).

Whether the expression of multiple mACHR subtypes on individual cells has important functional implications or reflects mere biological redundancy is unknown. We did not quantify the relative expression of each subtype in the present study. However, level of expression may not correlate with functional importance, a point supported by the observation that contraction in most smooth muscles is mediated by the M3 subtype, whereas the M2 subtype is quantitatively dominant (11, 12). The functional response we examined in the cultured cells was the ACh-induced rise in [Ca2+]i. We demonstrated that this rise in [Ca2+]i was caused mainly by the release of Ca2+ from intracellular stores. We anticipate that this effect could be mediated by the M1, M3, and/or M5 receptors because these mACHRs preferentially activate phosphoinositide hydrolysis and IP3-mediated release of Ca2+ from intracellular stores (11, 12). The potent effect of 0.01 μM 4-DAMP in blocking the ACh-induced rise in [Ca2+]i in our cells supports a dominant functional role for the M3 receptor in mediating this response. 4-DAMP also blocks the M1 receptor with a predicted receptor occupancy of 80% at the concentration tested. However, pirenzepine, which is M1 selective, should occupy 95% of M1 receptors and 44% of M3 receptors at a concentration of 0.1 μM. We observed a mean suppression of 35–39% of the ACh-induced [Ca2+]i transient with 0.1 μM pirenzepine, an effect consistent with partial blockade of the M3 receptor. Because we estimated that 0.1 μM methoctramine should block 89% of M2 receptors and 80% of M4 receptors, the observation of a mean suppression of 15–21% of the response by this antagonist makes it unlikely that either M2 or M4 receptors contributed substantially to this Ca2+ response. Moreover, these two subtypes preferentially couple to Gi-like G proteins and inhibit adenylate cyclase, a mechanism that is not thought to directly influence Ca2+ release from stores. Recent studies (34, 37) have shown that the M5 receptor activates a nonsel ective cation channel in smooth muscle but requires concurrent M3 activation, complicating the analysis of antagonist effects. The resulting depolarization would activate L-type Ca2+ channels, thus contributing to the [Ca2+]i response. Neither L-type channel activation by BAY K8644 nor antagonism by nifedipine had any effect on the ACh-induced [Ca2+]i transient in cultured human esophageal SMCs, further supporting our conclusion that the M5 receptor does not contribute to this acute response. However, we cannot rule out the possibility that M2 receptor activation may play a role in contributing to the plateau elevation of [Ca2+]i, observed with longer applications of ACh, because influx of extracellular Ca2+ contributes to this response.

We conclude that the ACh-induced Ca2+ transient in these cells is most likely due to the M3 receptor. However, a study of M3 and M5 mACHRs expressed in CHO-K1 cells has emphasized their similar pharmacologic profiles (35). It would be difficult to resolve their
relative contributions to a response even using detailed Scholz plot analysis with multiple antagonists (35). This approach is not feasible with the present experimental design, and thus we cannot rule out a contribution of the M3 receptor in the Ach-mediated [Ca^{2+}]_i transient.

This is the first report detailing the expression of mAChRs in human esophageal smooth muscle. Studies in humans have shown that atropine inhibits peristalsis in vivo (9) and blocks contraction of muscle strips in response to intrinsic nerve activation in vitro (32), indicating the involvement of mAChRs, but specific information regarding the mAChR subtypes involved is lacking. Using muscle strips and single cells acutely isolated from human esophagus, we (29) demonstrated that cholinergic excitation involves multiple signaling pathways, including the release of Ca^{2+} from intracellular stores. We inferred from these findings that M3 receptor-stimulated IP3 production was the most likely mechanism for triggering Ca^{2+} release. Moreover, we also have found that cholinergic contraction of human esophageal smooth muscle strips displayed a pattern of sensitivity to subtype-selective antagonists consistent with a M3 receptor-mediated response (26). Animal studies also support a major role for the M3 receptor subtype in mediating esophageal peristalsis in vivo (2, 15) and contraction of esophageal muscle strips in vitro (27). However, in dispersed CM cells of the cat esophagus, ACh-induced cell shortening showed a sensitivity to subtype-selective antagonists consistent with an M2-mediated response (30). These findings lead us to speculate that the functional contribution of mAChR subtypes in smooth muscle may depend on assay conditions or could be modulated by physiological factors. In light of the findings of the present study, the roles of multiple mAChR subtypes in human esophageal function will need to be reconsidered.

Certain properties we have described previously (29) for acutely isolated human esophageal cells and muscle strips are preserved in these SMCs in primary cultures. For example, both acutely isolated cells and cultured SMCs responded to ACh stimulation with a transient rise in [Ca^{2+}]_i that derives from release of Ca^{2+} from intracellular stores, consistent with a mechanism involving the IP3 signaling pathway and most likely involving the M2 (or M3) receptor. The findings were similar regardless of whether the cells were obtained from the LM or CM layer. In acutely isolated cells (29), a second plateau phase after the initial transient was observed and found to have a contribution from influx of extracellular Ca^{2+}. This plateau phase was also observed in cultured SMCs with prolonged application of ACh.

In summary, the present study demonstrates the feasibility of obtaining primary cultures of human esophageal SMCs with a high degree of purity and uniform characteristics suitable for in vitro experimentation. These cells were shown to express five known mAChR subtypes and to respond to cholinergic stimulation with a robust transient rise in [Ca^{2+}]_i. As in other smooth muscle types, the M3 receptor likely plays a major role in mediating this response, although additional functional roles for the remaining receptors cannot be ruled out based on our findings. The fact that these cells do not display a contractile response represents a potential limitation of this system. Nonetheless, the primary culture of esophageal SMCs we describe here may provide a useful additional model for the further study of human esophageal physiology by allowing opportunities for experimental intervention, otherwise limited by the obvious constraints imposed by studies involving human tissue.

We are grateful to T. Chrones and Y. Jiao for technical assistance, Dr. D. Driman for help with immunochemistry, and Drs. R. Inculet, R. Malthaner, and C. Rajgopal for providing esophagectomy specimens. This work was funded by the Medical Research Council of Canada. J. Wang is a Medical Research Council of Canada Postdoctoral Fellow and was in part supported by the Department of Medicine of the University of Western Ontario. H. G. Preiksaitis is an Ontario Ministry of Health Career Scientist.

REFERENCES


