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Quantitation of the Contractile Response Mediated by Two Receptors: M_2 and M_3 Muscarinic Receptor-Mediated Contractions of Human Gastroesophageal Smooth Muscle[S]

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ABSTRACT

Although muscarinic receptors are known to mediate tonic contraction of human gastrointestinal tract smooth muscle, the receptor subtypes that mediate the tonic contractions are not entirely clear. Whole human stomachs with attached esophagus were procured from organ transplant donors. Cholinergic contractile responses of clasp, sling, lower esophageal circular (LEC), midesophageal circular (MEC), and midesophageal longitudinal (MEL) muscle strips were determined. Sling fibers contracted greater than the other fibers. Total, M_2 and M_3 muscarinic receptor density was determined for each of these dissections by immunoprecipitation. M_2 receptor density is greatest in the sling fibers, followed by clasp, LEC, MEC, and then MEL, whereas M_3 density is greatest in LEC, followed by MEL, MEC, sling, and then clasp. The potency of subtype-selective antagonists to inhibit bethanechol-induced contraction was calculated by Schild analysis to determine which muscarinic receptor subtypes contribute to contraction. The results suggest both M_2 and M_3 receptors mediate contraction in clasp and sling fibers. Thus, this type of analysis in which multiple receptors mediate the contractile response is inappropriate, and an analysis method relating dual occupation of M_2 and M_3 receptors to contraction is presented. Using this new method of analysis, it was found that the M_2 muscarinic receptor plays a greater role in mediating contraction of clasp and sling fibers than in LEC, MEC, and MEL muscles in which the M_3 receptor predominantly mediates contraction.

Located at the junction of the tubular esophagus and the saccular stomach, the gastroesophageal junction (GEJ) is the area of transition from positive pressure in the abdominal cavity to the respiratory oscillations of negative and positive pressure in the thoracic cavity. It has the dual function of ensuring passage of a swallowed bolus and preventing gastroesophageal reflux. The existence of an anatomical sphincter at the GEJ has been disputed for more than half a century.

The existence of a sphincter at the GEJ was proposed in an observational and anatomical study of cadavers (Lerche, 1950). With the use of manometry in 1956, this same area was described as a high-pressure zone (HPZ) rather than an anatomical sphincter (Code et al., 1956). Since then, much has been written about the HPZ in the lower esophagus. It is mainly composed of pressures from the extrinsic crural diaphragm and the intrinsic muscles of the stomach and the lower esophagus (McCray et al., 2000).

In 1979, the arrangement of the smooth muscles around the GEJ was first described as consisting of "clasp" fibers at the lesser curvature and "sling" fibers at the greater curvature of the stomach, suggesting that these muscle fibers might produce the HPZ at the GEJ (Liebermann-Meffert et al., 1979). This theory laid the foundation for further studies on the physiology, pathology, and pharmacology of the GEJ. Over the span of the next three decades, the formation and regulation of the HPZ were closely studied with the help of high-resolution endoscopic ultrasound, esophageal manometry, autopsies, and animal experiments (Burleigh, 1979; McCray et al., 2000). Differences have been reported between

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ABBREVIATIONS: GEJ, gastroesophageal junction; HPZ, high-pressure zone; EFS, electric field stimulation; LES, lower esophageal sphincter; LEC, lower esophageal circular; MEC, midesophageal circular; MEL, midesophageal longitudinal; QNB, quinuclidinyl benzilate; TE, Tris-EDTA; TEDC, Tris-EDTA buffer containing 1% digitonin and 0.2% cholic acid; PLC, phospholipase C; DAR, darifenacin; METH, methoctramine; BETH, bethanechol; CRC, concentration response curve.

human clasp and sling stomach muscle fibers in the sensitivity and maximal responses to acetylcholine, dopamine, phenylephrine, and isoproterenol (Tian et al., 2004). Differences in response to electric field stimulation (EFS) have also been reported between both the clasp and sling fibers shown to relax to EFS, whereas the areas caudal to this (stomach) contracted under EFS (Burleigh, 1979).

Muscarinic receptors belong to the G protein-coupled receptor family. Five subtypes designated M_1 to M_5 exist. No completely specific agonists or antagonists are known for any of the subtypes; however, some very specific toxins have been identified. The potency of subtype-selective muscarinic receptor antagonists suggests that in most smooth muscles, contraction is primarily mediated by the M_3 receptor subtype (Caulfield, 1993; Caulfield and Birdsall, 1998). M_2-mediated contractile responses have been shown in smooth muscle cells isolated from the cat esophagus (Biancani et al., 1997). However, M_3 receptors predominantly mediate contraction of smooth muscle cells isolated from the cat lower esophageal sphincter (LES) circular smooth muscle. In an experimental model of esophagitis created by perfusing the esophagus with HCl, the affinity of antimuscarinic drugs is altered and is intermediate between their reported M_2 and M_3 affinities (Biancani et al., 1994).

Under certain experimental conditions, several studies have shown that the M_2 receptor subtype contributes to the contractile response. These include alkylation of M_3 receptors with increased intracellular levels of cAMP in the rat bladder (Hegde et al., 1997; Braverman and Ruggieri, 1999), guinea pig ileum (Ehlert and Thomas, 1995), and trachea (Thomas and Ehlert, 1996) or after alkylation without increasing intracellular cAMP levels in other tissues such as the guinea pig gallbladder (Braverman et al., 2000) and colon (Sawyer and Ehlert, 1998). In some experimentally induced pathologies, an increased contractile role for the M_2 receptor subtype is evident. These include a cat model of experimentally induced esophagitis (Sohn et al., 1997), the denervated rat bladder (Braverman et al., 1998), and a model of acute cholecystitis in the guinea pig gallbladder (Braverman et al., 2000).

The aim of the present study was to determine which muscarinic receptors mediate contraction of human clasp, sling, LEC, MEC, and MEL muscle fibers and to quantify the density of total and M_2 and M_3 muscarinic receptor subtypes in these tissues. This information may suggest useful targets for the development of drugs to treat disorders of the GEJ.

Materials and Methods

Materials. All drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO), except darifenacin (which was a generous gift from Pfizer Central Research, Sandwich, Kent, UK), digitonin (Wako Pure Chemicals, Osaka, Japan), and pansorbin (Calbiochem, San Diego, CA).

Human stomachs with the attached esophagus were obtained, with consent, from brain-dead organ transplant donors through either the National Disease Research Interchange (Philadelphia, PA) or the International Institute for the Advancement of Medicine (Jessup, PA). Peritoneal fat was removed, and dissection began using microscissors to remove the most superficial longitudinal fibers in a circular pattern around the esophagus. The deeper circular fibers were removed next, moving from the greater curvature toward the lesser curvature. The exact location of the sling and clasp fibers was identified at the greater and lesser curvature of GEJ, respectively, once the superficial longitudinal fibers were removed. Sling muscle fibers were removed from a relatively straight section of the greater curvature. Clasp fibers were obtained 2 to 3 cm distal to GEJ along the lesser curvature. The LEC fibers were obtained from the thickened area of the esophagus approximately 1 to 2 cm proximal to the stomach. The MEC and MEL fibers were obtained from the esophagus 10 cm proximal to the stomach. The muscles were further divided into individual strips, each measuring 1 to 2 mm in width and 8 to 10 mm in length. Care was taken to ensure the orientation of the muscle fibers parallel to the muscle strips. The muscle strips were then suspended with 0.5 g of tension in tissue baths containing 10 ml of modified Tyrode's solution (125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 23.8 mM $NaHCO_3$, and 5.6 mM glucose) and equilibrated with 95/5% O_2/CO_2 at 37°C.

Bethanechol Response Curves. After equilibration to the bath solution for 30 min, the strips were incubated for 30 min in the presence or absence of one of three concentrations of the competitive M_2-selective antagonist methoctramine (1E-7, 1E-6, or 1E-5 M) or the competitive M_3-selective antagonist darifenacin (3E-8, 1E-7, or 3E-7 M). Dose-response curves were derived from the peak tension developed after the cumulative addition of nonsubtype-selective muscarinic receptor agonist bethanechol. Bethanechol concentrations at half-log intervals from 1E-8 up to 1E-2 M if required to reach maximal contraction were used with approximately 3 min between addition of successive concentrations. Either vehicle or one concentration of methoctramine or darifenacin was used for each muscle strip. Dose ratios were determined based on the average of the responses of vehicle (H_2O)-treated strips. EC_{50} values were determined for each strip using a sigmoidal curve fit of the data (Origin; OriginLab Corp., Northampton, MA), and Schild plots were constructed.

Immunoprecipitation. Immunoprecipitation of muscarinic receptors from the individual dissections was performed using subtype-selective antibodies. The specificity of these antibodies and methods has been described in detail previously (Braverman et al., 2007). This immunoprecipitation assay makes use of tandem specificity: the specificity of [^{3}H]QNB binding to only muscarinic receptors and the specificity of the individual antibody binding to only the given subtype. If the antibody binds to other proteins that do not bind [^{3}H]QNB, then those proteins would not be detected in the assay. Likewise, if [^{3}H]QNB binds to other proteins that do not bind to the antibody, then those proteins would not be detected by the assay. In brief, the tissues were homogenized at 100 mg/ml in ice-cold Tris-EDTA (TE) buffer, with 10 μg/ml of the following protease inhibitors: soybean and lima bean trypsin inhibitors, aprotinin, leupeptin, pepstatin, and α2-macroglobulin. Twenty microliters of the nonsubtype-selective muscarinic receptor antagonist [^{3}H]QNB (49 Ci/mM, approximately 4000 cpm/μl) per milliliter of assay homogenate was added and incubated at room temperature for 30 min, with inversion every 5 min. Samples were pelleted via centrifugation at 20,000*g* for 10 min at 4°C, and the pellet was solubilized in TE buffer containing 1% digitonin and 0.2% cholic acid (1% TEDC), with the above-mentioned protease inhibitors at 100 mg of wet weight per ml. Samples were incubated for 50 min at 4°C, with inversion every 5 min, and then centrifuged at 30,000*g* for 45 min at 4°C. The supernatant containing the solubilized receptors was incubated overnight after addition of the M_2 antibody, the M_3 antibody, or vehicle at 4°C.

To determine total receptor density, samples were desalted over Sephadex G-50 minicolumns with 0.1% TEDC. M_2 and M_3 receptors were precipitated by adding 200 μl of pansorbin and incubated at 4°C for 50 min, with inversion every 5 min. The precipitated receptors were pelleted via centrifugation at 15,000*g* for 1 min at 4°C, and the pellet was surface washed with 500 μl of 0.1% TEDC. Fifty microliters of 72.5 mM deoxycholate/750 mM NaOH was added and incubated for 30 min at room temperature. The pellet was resuspended in 1 ml of TE buffer and neutralized with 50 μl of 1 M HCl. Radioactive counts were determined by liquid scintillation spectrometry. Protein

content was determined by a Coomassie Blue dye binding protein assay using bovine serum albumin as a standard. Receptor density (mean ± S.E.M.) is reported as femtomoles of receptor per milligram of solubilized protein.

Statistics. All statistical differences were determined by a nonparametric statistic (Wilcoxon rank sum/Mann-Whitney U test) because of nonhomogenous variances.

Results

Immunoprecipitation. Five different dissections of human gastroesophageal smooth muscle were studied. These sections were clasp, sling, LEC, MEL, and MEC. For each dissection, we determined total, M_2, and M_3 muscarinic receptor densities using immunoprecipitation (as described under *Materials and Methods*), and we did this as a prelude to subsequent studies of bethanechol-induced contraction, which are also described below. The results of the receptor density determinations are shown in Table 1. The rank order of total receptor density in the five different smooth muscle dissections was sling > LEC > clasp > MEL ≈ MEC fibers. The M_2 receptor subtype density followed a similar pattern as total receptor density with sling > clasp > LEC > MEC ≈ MEL fibers. However, the M_3 receptor subtype density was 60 to 83 fmol/mg protein for the sling, LEC, MEC, and LEC fibers but approximately 10-fold less (8 ± 2 fmol/mg protein) for the clasp fibers.

Concentration-Effect Relationships. Representative tracings of bethanechol concentration-response experiments for each muscle fiber are shown in Fig. 1. Spontaneous activity was sometimes observed; however, this did not interfere with the determination of the effect of bethanechol, and the antagonists had no effect on baseline spontaneous activity. Each muscle section was studied for isometric tension development in response to bethanechol and each demonstrated a dose-related response to this agonist. For example, Fig. 2 shows the graded concentration-effect relationship for bethanechol in clasp fibers. Also shown in this figure are the curves for graded doses of this agonist with three different fixed concentrations of darifenacin, a relatively selective M_3-competitive antagonist. Shown in Fig. 3 are the curves for graded doses of this agonist with no antagonist and with two different fixed concentrations of methoctramine, a relatively selective M_2-competitive antagonist. The fitted curves show

TABLE 1

Total, M_2, and M_3 muscarinic receptor density (femtomoles per milligram of solubilized protein) for different dissections of human GEJ muscles

Total muscarinic receptor density was determined by total [^{3}H]QNB binding, whereas M_2 an M_3 receptor density was determined using subtype-selective immunoprecipitation (as described under *Materials and Methods*). Results are reported as mean ± S.E.M. for at least duplicate determinations from two individual organs for clasp and sling fibers, whereas n = 3 donors for LEC, MEL, and MEC fibers. Statistical differences were determined using nonparametric statistics with a Mann-Whitney U test.

Muscle	Total	M_2	M_3	M_2/M_3 Ratio
Clasp	228 ± 20**a**	116 ± 16b,c,**d**	8 ± 2**a,b,c,d**	14.5
Sling	353 ± 7**b,c,d**	171 ± 6**b,c,d**	60 ± 14	2.85
LEC	244 ± 12**c,d**	73 ± 7	83 ± 13	0.88
MEC	190 ± 7	59 ± 9	69 ± 9	0.86
MEL	209 ± 10	54 ± 4	78 ± 3	0.69

($P < 0.05$ if not bold, and $P < 0.01$ if bold).
a, Significantly different from sling fibers.
b, Significantly different from LEC fibers.
c, Significantly different from MEC fibers.
d, Significantly different from MEL fibers.

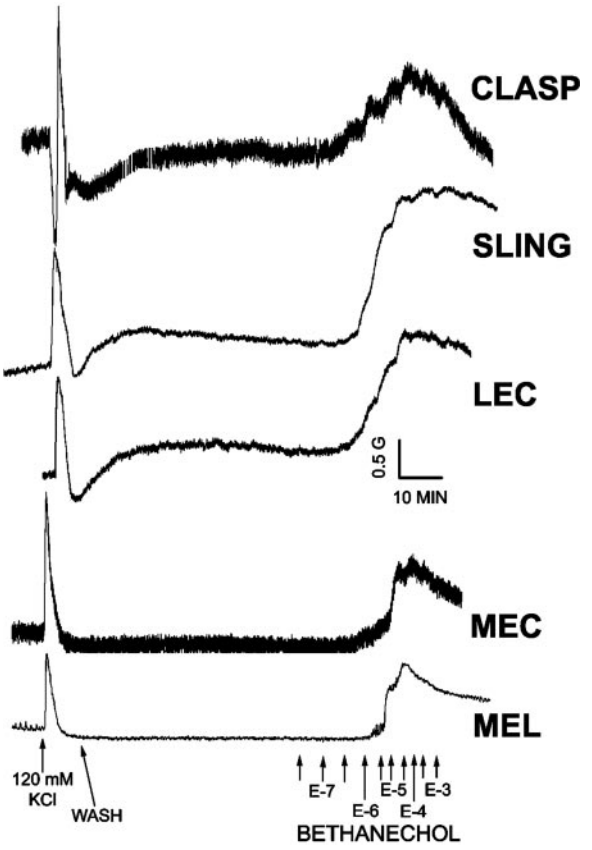

Fig. 1. Original tracings of bethanechol concentration-response experiments from the various smooth muscle components of the human GEJ.

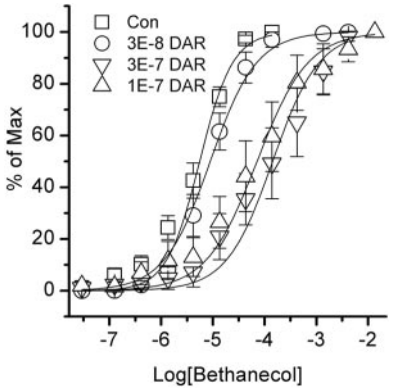

Fig. 2. Concentration-response curves for bethanechol-induced contraction of human clasp fibers in the presence of various concentrations of darifenacin (DAR). Inhibition of bethanechol induced human clasp fiber contractions with increasing concentrations of the M_3-selective antagonist darifenacin causes parallel rightward shifts in the concentration-response curve. Results are shown as percentage of the maximal response shown in Table 2. Control, n = 14 strips from four donors; 30 nM DAR, n = 6 strips from two donors; 100 nM DAR, n = 5 strips from two donors; and 300 nM DAR, n = 7 strips from two donors.

an obvious dose-dependence; furthermore, they also show rightward shifts resulting from each antagonist dose. These log plots show approximate parallelism (indicative of competitive inhibition). However, the relatively low potency calculated by Schild analysis for darifenacin in clasp fibers (pA_2 = 7.8 ± 0.2) compared with the reported darifenacin affinity at M_3 receptors (pK_b = 8.65) and M_2 receptors (pK_b = 7.20)

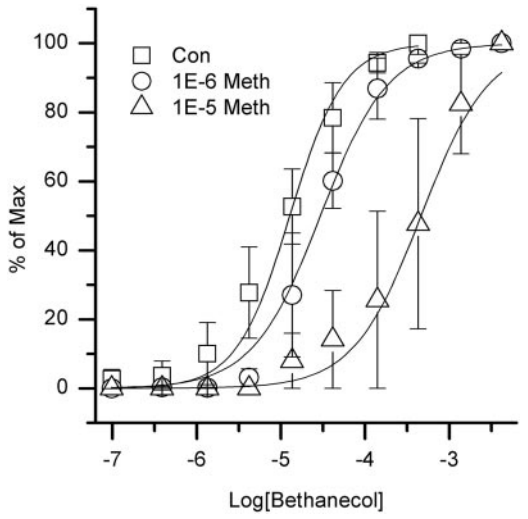

Fig. 3. Concentration-response curves for bethanechol-induced contraction of human clasp fibers in the presence of various concentrations of methoctramine (METH). Inhibition of bethanechol induced human clasp fiber contractions with increasing concentrations of the M_2-selective antagonist methoctramine causes parallel rightward shifts in the concentration-response curve. Results are shown as percentage of the maximal response shown in Table 2. Control, $n = 14$ strips from four donors; 1 μM METH, $n = 3$ strips from one donor; and 10 μM METH, $n = 3$ strips from one donor.

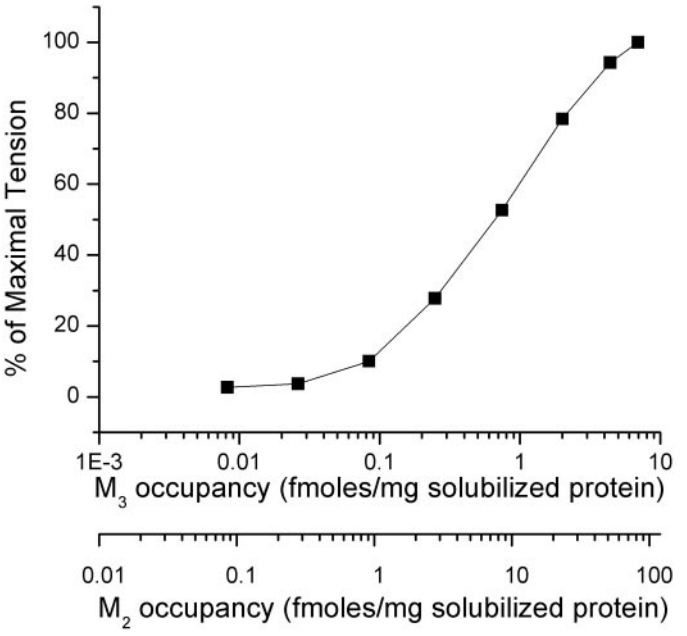

Fig. 4. Bethanechol-induced clasp fiber contraction as a function of M_2 and M_3 receptor occupancy. The human clasp fiber bethanechol concentration-response curve was converted into occupation response curves for the M_2 and the M_3 receptor subtypes. The *y*-axis is the percentage of the maximal bethanechol effect, and the lower *x*-axis shows the density of M_2 receptor occupied by bethanechol, whereas the upper *x*-axis shows the density of M_3 receptors occupied. Receptor occupation = [A]/([A] + K_A), where [R] denotes the receptor concentration (R was obtained from the immunoprecipitation results shown in Table 1), K_A is the agonist dissociation constant (reciprocal of affinity), and [A] is the agonist concentration. For this purpose, we used published values of K_A (Evans et al., 1985; McKinney et al., 1991) for bethanechol as follows: K_A for M_2 = 170 μM and K_A for M_3 = 110 μM.

suggests that M_2 receptors mediate contraction. In contrast, the low potency calculated for methoctramine in clasp fibers (pA_2 = 6.3 ± 0.2) compared with its reported affinity at M_2 receptors (pK_b = 8.05) and M_3 receptors (pK_b = 6.60) suggests that M_3 receptors mediate contraction (Caulfield, 1993; Caulfield and Birdsall, 1998). Darifenacin potency (pA_2) calculated by Schild analysis is 8.0 ± 0.1, 8.2 ± 0.2, 8.2 ± 0.1, and 8.4 ± 0.2, and methoctramine potency (pA_2) is 6.8 ± 0.2, 6.2 ± 0.2, 5.7 ± 0.2, and 5.6 ± 0.3 in sling, LEC, MEC, and MEL fibers, respectively.

These potencies in clasp and sling fibers suggest that the bethanechol effect is mediated by both M_2 and M_3 receptors; hence, using Schild plot analysis that is based on the assumption that one receptor is mediating the effect is inappropriate. For that reason, and to add clarity to the relative contribution of each receptor subtype, we transformed each bethanechol concentration to receptor occupations of both M_2 and M_3 receptors. That transformation is based on mass-action binding which, at equilibrium, gives receptor occupation = [A][R]/([A] + K_A), where [A] denotes the agonist concentration, [R] is the receptor concentration, and K_A is the agonist dissociation constant (reciprocal of affinity). For this purpose, we used published values of K_A for bethanechol as follows: K_A for M_2 = 1.7 E-4 M derived using cloned human M_2 receptors expressed in Chinese hamster ovary cells (McKinney et al., 1991) and K_A for M_3 = 1.1 E-4 M derived from human astrocytoma cells, which predominantly express M_3 receptors (Evans et al., 1985). The concentration-effect curve in clasp fibers is shown Fig. 4 in which the abscissa scales show the simultaneous values of M_2 and M_3 occupancy that follow from the bethanechol concentrations that were used. It is noted that the M_2, M_3 occupation pair that gives 50% of the maximum tension is the pair (8.8, 0.9). However, from this graph it is not apparent that occupancy of both M_2 and M_3 receptors occurs simultaneously, resulting in contraction. This critical point is more clearly evident in an alternative

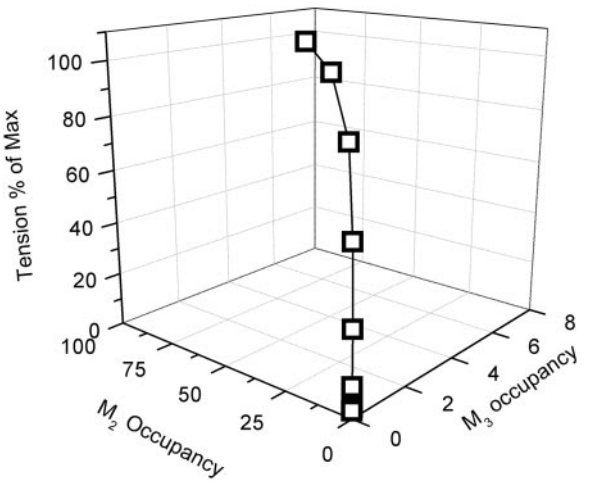

Fig. 5. Three-dimensional graph of bethanechol-induced clasp fiber contraction as a function of M_2 and M_3 receptor occupancy.

view of this dual receptor occupation-effect (Fig. 5), which is a three dimensional plot with the effect shown as the height above the M_2-M_3 occupation plane.

Antagonist Effects. The presence of a fixed concentration of a competitive antagonist reduces the agonist occupancy to a lower quantity given by the equilibrium equation of Gaddum (1937): receptor occupation = [A][R]/[A] + K_A(1 + [B]/K_B), where [B] is the antagonist concentration and K_B is its dissociation constant. Of course, this holds at each recep-

tor with each receptor's applicable values of K_A and K_B. Thus, the presence of the antagonist yields bethanechol occupancy at M_2 and M_3, each calculated from the above-mentioned equation, thereby giving a view of occupation of this receptor pair and its corresponding effect. This relation is shown in the three-dimensional plot (Fig. 6). This graph, for clasp fibers, was generated using published affinity values (Caulfield, 1993; Caulfield and Birdsall, 1998), from three different doses of darifenacin (pK_B M_3 = 8.65, pK_B M_2 = 7.2, thus relatively selective for M_3) and two different doses of methoctramine (pK_B M_3 = 6.6, pK_B M_2 = 8.1, thus relatively selective for M_2). The use of the two antagonists in several different fixed concentrations yielded an array of M_2, M_3 occupancy values and their associated effects.

A more global view of these results is provided in the form of a response surface, also shown in Fig. 6, indicating that both M_2 and M_3 receptors have a significant role in mediating contraction in clasp fibers. This is based on the occupancy-effect relationship in the presence of the antagonists. In the presence of darifenacin, where very few M_3 receptors are occupied by bethanechol, the occupancy-effect relationship is more dependent on M_2 occupancy than on M_3 occupancy. This can be seen on the surface plot in Fig. 6 where the occupancy effect curve in the presence of darifenacin is almost parallel with the axis of M_2 occupancy and shows very little deflection along the M_3 occupancy axis. In contrast, in the presence of methoctramine, where very few M_2 receptors are occupied by bethanechol, the occupancy-effect relationship is more dependent on M_3 occupancy than on M_2 occupancy.

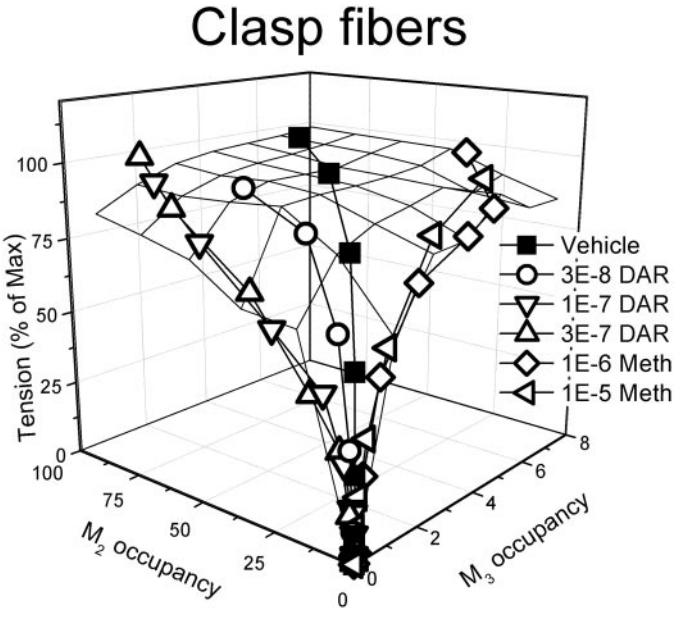

Fig. 6. Surface plot of clasp fiber contraction as a function of M_2 and M_3 receptor occupancy. Subtype-selective antagonists alter the number of M_2 and M_3 receptors occupied by bethanechol that yield a given effect level. Using the formula for occupancy of an agonist in the presence of an antagonist [receptor occupancy = $AR/(A + K_a(1 + B/K_b))$] and published antagonist affinity values (Caulfield, 1993; Caulfield and Birdsall, 1998), the M_2 and M_3 occupancy-effect curves in the presence of three concentrations of darifenacin and two concentrations of methoctramine were derived. A surface plot showing the effect of combinations of M_2 and M_3 occupancy in human clasp fibers is overlaid. The surface plot was constructed by transformation of the individual data points into a matrix using a random gridding method with Kringing correlation (Origin, OriginLab Corp.).

Other Gastrointestinal Muscle Fibers. The analysis of occupancy-effect relations described above for the clasp fibers was also conducted on the human sling, LEC, MEC, and MEL smooth muscle fibers. For each muscle group, a surface plot, similar to that of the clasp fibers, was generated. The surface plot for sling fibers (Supplemental Fig. 1), which have more M_2 receptors than M_3 receptors (Table 1), is similar to the surface plot for clasp fibers, which also have more M_2 than M_3 receptors. The surface plot for LEC fibers, which have more M_3 receptors than M_2 receptors, has a different shape (Fig. 7). The surface plots for MEC and MEL fibers, which also have more M_3 receptors than M_2 receptors, are similar to that for LEC fibers (Supplemental Figs. 2 and 3). In these muscle groups, the occupation-effect relationships demonstrate that contraction is more dependent on M_3 occupation than M_2 receptor occupation. This is demonstrated by the occupation-effect relationship of the LEC fibers shown in Fig. 7. When the M_2-selective antagonist methoctramine is present, the occupation-effect relationship shows that contraction is dependent on occupation of M_3 receptors. In addition, in the presence of darifenacin, contraction increases with increasing M_2 occupancy, but only up to a point; maximal tension is only obtained when the bethanechol concentration is high enough to compete for occupation of the M_3 receptors. This is demonstrated in Fig. 7 at the point labeled "a" by the deflection to the right, which means increasing M_3 occupancy, of the occupation-effect curve in the presence of darifenacin.

Discussion

The arrangement of the clasp/sling muscle fiber complex was first described in 1979 (Liebermann-Meffert et al., 1979) and was hypothesized to be a physiologic circular smooth muscle sphincter in the distal esophagus (Code et al., 1956). However, until recently, no intrinsic muscarinic receptor-mediated pressure in the proximal stomach has been demonstrated from the gastric sling/clasp fiber muscle complex.

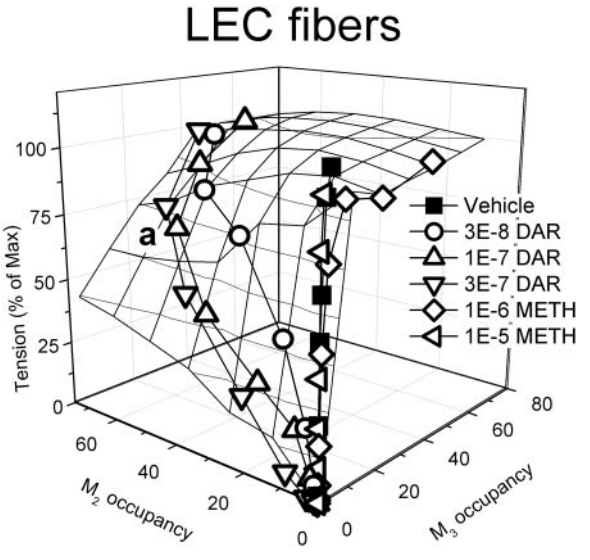

Fig. 7. Surface plot of LEC fiber contraction as a function of M_2 and M_3 receptor occupancy. The surface plot was constructed as described for Fig. 6. The point labeled as a denotes the rightward deflection toward increasing M_3 occupancy for contraction of LEC fibers in the presence of the M_3-selective antagonist darifenacin.

Along with this pressure generated, we observed a second muscarinic receptor-mediated pressure profile in the distal esophagus associated with the LEC. These distinct pressure profiles were discovered using simultaneous ultrasound and manometry (Brasseur et al., 2007). Thus, the importance of muscarinic tone within both the distal clasp/sling muscle fiber complex and the more proximal LEC is established.

Using the same techniques in gastroesophageal reflux disease patients, we found that the proximal pressure profile due to the LEC was present. However, the gastric sling/clasp fiber pressure profile was absent in all gastroesophageal reflux disease patients (Miller et al., 2009). Thus, our previous study demonstrated the importance of the intrinsic muscarinic gastric sling/clasp muscle fiber pressure profile to the antireflux barrier. Given the importance of these two distinct muscle complexes to the antireflux barrier, it is important to understand how these muscle complexes function. This includes any anatomic or physiologic differences between the muscle groups that generate the pressure to prevent reflux and the rest of the smooth muscles within the esophagus that do not contribute to the antireflux barrier. It was with these goals in mind that we undertook the current study.

The results presented above show that the density of muscarinic receptor subtypes is different in the different smooth muscle preparations of the human GEJ and esophagus. Both the clasp and the sling fibers, which work together to contract the GEJ to prevent reflux have a greater density of M_2 than of M_3 receptors similar to most other smooth muscles studied. In LEC, MEC, and MEL fibers, however, M_3 receptor density is greater than M_2 receptor density.

The bethanechol-induced maximal contraction is greater in the sling fibers than all other fibers studied (Table 2). This result is in general agreement with a previous study showing that human sling fibers contract significantly greater to acetylcholine than human clasp fibers (Tian et al., 2004). As shown here for the first time, sling fibers may contract greater than the other fibers because they have a greater total density of muscarinic receptors than all other muscles studied.

Classical pharmacologic analysis of concentration-effect relationships was formulated before the concept of multiple receptor subtypes existed and is based upon the assumption that one receptor mediates one effect. Because of this assumption, there is no theoretical framework that allows Schild analysis to yield meaningful conclusions in the context of multiple receptors mediating a response. Schild analysis yielded conflicting conclusions with respect to which receptor subtype mediates contraction of clasp and sling fibers. The M_3-selective antagonist darifenacin yielded an affinity intermediate between that reported for M_2 and M_3 receptors, suggesting that both receptors may mediate the contractile response. However, the M_2-selective antagonist methoctramine yielded a low affinity, suggesting that M_3 receptors mediate contraction. Thus, no definitive conclusions regarding the contribution of each subtype to the contractile response could be drawn using Schild analysis.

If contraction was mediated by purely M_3 receptors, we would expect that darifenacin would have high potency and methoctramine would have low potency. Conversely, if the M_2 receptor solely mediated contraction, we would expect that darifenacin would have relatively low potency, whereas methoctramine would be highly potent. The apparently contradictory results we obtained can be explained if both M_2 and M_3 receptor subtypes mediate contraction. The M_3-selective antagonist darifenacin has only minimal effects until its concentration is high enough to block M_2 receptors in addition to M_3 receptors. The M_2 subtype mediates contraction when low concentrations of darifenacin block M_3 receptors, thus there is very little to no shift in the CRC in the presence of low concentrations of darifenacin. Because the rightward shifts in the CRCs in the presence of high concentrations of darifenacin are due mostly to occupation of M_2 receptors, the result is parallel CRCs. The opposite is also true for methoctramine, in which M_3 receptors mediate contraction until the methoctramine concentration is high enough to block both M_2 and M_3 receptors.

Using muscarinic receptor knockout mice, we have previously shown that contraction of the stomach body is mediated by both M_2 and M_3 receptor activation in an additive manner (Braverman et al., 2008). The M_3 receptor alone can mediate a maximal cholinergic contraction; however, M_2 receptors alone can only mediate a contraction of approximately 45% of M_3 receptors. The type of analysis used in that report is dependent on determining the contribution of the individual receptor subtypes, as can be determined in receptor knockout mice. However, because of the lack of completely specific antagonists, the contribution of the individual receptor subtypes cannot be determined in human tissue. Thus, we constructed three-dimensional occupation-effect graphs to allow visualization of how agonist occupancy of M_2 and M_3 receptors relates to contraction. Based on this analysis, both M_2 and M_3 receptor subtypes are involved in mediating contraction of all muscle fibers studied here. However, in clasp and sling fibers, where the M_2 receptor subtype is more abundant than the M_3 receptor subtype, M_2 receptors have a greater contribution to the contractile response than in the LEC, MEC, and MEL. In the LEC, MEC, and MEL fibers in which the M_3 receptor subtype is more abundant than the M_2 receptor subtype, M_3 receptors have a greater contribution to the contractile response.

Analysis of the surface plot for the clasp fibers demonstrates that M_2 and M_3 occupancy is 8.8 and 0.9 fmol/mg solubilized receptor, respectively, at 50% of the maximal contraction. M_2 density in clasp fibers is 116 fmol/mg solubilized receptor; thus, 7.6% (8.8/116) of all M_2 receptors are occupied by agonist at the 50% effect level. M_3 density in clasp fibers is 8 fmol/mg solubilized receptor; so, 11.3% (0.9/8) of all M_3 receptors are occupied by agonist at the 50% effect

TABLE 2
Maximal tension and bethanechol potency determined for the different dissections of human GEJ muscles
Results are reported as mean ± S.E.M. Statistical differences were determined using nonparametric statistics with a Mann-Whitney U test.

Muscle	BETH Max	BETH pEC_{50}
	G	
Clasp	1.20 ± 0.17a,**c** (n = 14)	5.08 ± 0.09**c** (n = 14)
Sling	2.18 ± 0.24**b,c,**d (n = 37)	4.98 ± 0.10**b,c** (n = 37)
LEC	0.92 ± 0.09 (n = 29)	5.19 ± 0.11**c** (n = 29)
MEC	0.79 ± 0.07d (n = 24)	4.34 ± 0.08d (n = 24)
MEL	1.37 ± 0.21 (n = 10)	4.80 ± 0.09 (n = 10)

$P < 0.05$ if not bold, and $P < 0.01$ if bold.
a, Significantly different from sling fibers.
b, Significantly different from LEC fibers.
c, Significantly different from MEC fibers.
d, Significantly different from MEL fibers.

level, a percentage similar to M_2 receptors. Based on these occupancies, it seems there is a relatively large surplus of both M_2 and M_3 receptors in human clasp fibers. In the presence of high concentrations of darifenacin (1E-7 M), the percentage of M_2 receptor subtypes occupied by bethanechol is increased compared with the percentage of M_3 receptors occupied. Here, M_2 and M_3 occupancy at 50% of maximal contraction is 33.5% (38.9/116) and 4.25% (0.32/8), respectively, suggesting that there is a relatively small surplus of M_2 receptors. Conversely, in the presence of methoctramine (1E-6 M), M_2 and M_3 occupancy at 50% of maximal contraction is 0.26% (0.3/116) and 7.5% (0.6/8), respectively, suggesting that there is a relatively large surplus of M_3 receptors.

The contribution of each receptor subtype is probably dependent not only on the density of the individual subtypes but also on which intracellular signal transduction mechanisms are activated by each subtype. For example, in the feline esophagus, where the M_2 receptor subtype predominantly mediates contraction, the M_2 receptor activates phosphoinositide-PLC, phosphatidylcholine-PLC, phospholipase D, and cytosolic phospholipase A_2 to produce contraction dependent on a protein kinase C. However, in the cat LES, similar to the human LEC reported here, the M_3 receptor subtype primarily mediates contraction. In the cat LES, this contraction is mediated by activation of PLC, generation of inositol trisphosphate, release of intracellular calcium, activation of calmodulin, and finally activation of myosin light chain kinase. (Harnett et al., 1999). As demonstrated here, the contribution of M_2 and M_3 receptors is not the same in the different human smooth muscle preparations. In the cat esophagus, the M_2 receptor subtype predominantly mediates contraction, but as demonstrated here, the M_3 receptor predominantly mediates contraction in the human longitudinal and circular esophageal muscle layers. Thus, contribution of the individual subtypes to contraction of the same smooth muscles can be different between species.

In summary, the receptor density of each smooth muscle group within the esophagus differs according to the muscle location and function. Using a new method of analysis, it was found that both the M_2 and the M_3 receptors contribute to the tonic contraction of all of the smooth muscles of the esophagus. However, the M_2 muscarinic receptor plays a greater role in mediating contraction of sling and clasp fibers than in LEC, MEC, and MEL muscles in which the M_3 receptor predominantly mediates contraction.

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