Comparison of Human and Porcine Gastric Clasp and Sling Fiber

Contraction by M₂ and M₃ Muscarinic Receptors

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Running head: M₂ & M₃ mediated contraction of pig clasp & sling fibers

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Abbreviations: cyclic adenosine monophosphate (cAMP), concentration response curve (CRC), darifenacin (DAR), gastroesophageal junction (GEJ), high pressure zone (HPZ), knockout (KO), lower esophageal circular (LEC), lower esophageal sphincter (LES), methoctramine (METH), mid esophageal circular (MEC), mid esophageal longitudinal (MEL), 10 mM Tris-1mM EDTA pH 7.5 (TE), 1% digitonin and 0.2% cholic acid in TE (1% TEDC).
Abstract:

To compare the gastroesophageal junction of the human with the pig, M2 and M3 receptor densities and the potencies of M2 and M3 muscarinic receptor subtype selective antagonists were determined in gastric clasp and sling smooth muscle fibers. Total muscarinic and M2 receptors are higher in pig than human clasp and sling fibers. M3 receptors are higher in human compared to pig sling fibers but lower in human compared to pig clasp fibers. Clasp fibers have fewer M3 receptors than sling fibers in both humans and pigs. Similar to human clasp fibers, pig clasp fibers contract significantly less than pig sling fibers. Analysis of the methoctramine Schild plot suggests that M2 receptors are involved in mediating contraction in pig clasp and sling fibers. Darifenacin potency suggests that M3 receptors mediate contraction in pig sling fibers and M2 and M3 receptors mediate contraction in pig clasp fibers. Taken together, the data suggests that both M2 and M3 muscarinic receptors mediate the contraction in both pig clasp and sling fibers similar to human clasp and sling fibers.

Key words: gastro esophageal reflux, receptor immunoprecipitation, smooth muscle contractility, animal models.
Introduction:

Gastroesophageal reflux disease (GERD) is a common disease with an incidence of approximately 5 per every 1000 person-years and prevalence estimated to be 10-15% in the adult Western population (8). GERD is a major health care burden as regards to the number of doctor visits, the number of work days lost, and the billions of dollars spent on prescription and over the counter medications. The pathophysiologic events occurring in GERD appear at first to be simple, since the underlying pathogenesis is the movement of gastric contents into the esophageal lumen. However, there is an intense debate surrounding GERD’s pathogenesis with respect to how the protective mechanisms at the gastro esophageal junction high pressure zone (GEJHPZ) function and fail.

Initially, the proposed barrier between the lower esophagus and the stomach was thought to be an anatomical sphincter (16). With the advent of manometry and high resolution endoscopic ultrasonography, a high pressure zone (HPZ) was recognized (12). A detailed description of the anatomical arrangement of the smooth muscle fibers around the gastroesophageal junction (GEJ) was published in 1979. This study reported the muscle fibers at the lesser curvature of the stomach to be clasp muscle fibers and those at the greater curvature to be sling muscle fibers. The suggestion was proposed that these fibers might be responsible for the HPZ at the GEJ (17). Once this assertion was made, further studies were designed to determine the physiologic, pathologic, and pharmacologic attributes of these structures. High resolution endoscopic ultrasound, esophageal manometry, autopsy, and animal experiments have been utilized over the past three decades in order to more fully document the role of the
gastric sling/clasp muscle fiber complex in the formation and regulation of the HPZ (7, 18). Important results include the differences reported in the sensitivity and maximal responses to acetylcholine, dopamine, phenylephrine, and isoproterenol by clasp stomach fibers versus sling stomach fibers (22). The clasp and sling fibers were also shown to relax to electric field stimulation, while areas caudal to this contracted.

The macroscopic arrangement of lower esophageal (LE) smooth muscle fibers in humans and pigs is similar. There are short transverse muscle clasps on the lesser curvature of the stomach (clasp fibers) and long oblique gastric sling fibers on the greater curvature of the stomach (17, 23). Because of this similarity the porcine model has been used as a representative model of the human GEJ (15, 21). The size, anatomical transverse asymmetry (clasp and sling fibers), histology (smooth muscle cells), organization of the enteric nervous system, neurotransmitters, and the enteric motor neurons (EMNs) are similar to those of humans (1, 6). In vivo studies in pigs have shown a swallow-induced lower esophageal sphincter relaxation followed by contractions similar to that in humans (23).

The arrangement of the clasp/sling muscle fiber complex was first described in 1979 (17). However, until recently, no intrinsic muscarinic receptor mediated pressure in the distal esophagus has been demonstrated to arise specifically from the gastric sling/clasp fiber muscle complex in-vivo. We identified a distinct pressure profile from the gastric sling/clasp muscle fiber complex using simultaneous high resolution ultrasound and manometry with pharmacologic manipulation using atropine in normal control subjects (2). Along with the pressure generated by the clasp/sling muscle fiber complex, a second, more proximal muscarinic pressure profile associated with the LEC
muscle was also seen. 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 GERD patients, we found that the proximal pressure profile due to the LEC was present. However, the gastric sling/clasp muscle fiber pressure profile was absent (20). This establishes the importance of the intrinsic muscarinic gastric sling/clasp muscle fiber pressure profile to the antireflux barrier.

Considering their importance in the GEJHPZ, detailed investigations of these muscles is important in delineating the pathophysiology of GERD. This includes any anatomic or physiologic differences between the muscles that generate the pressure to prevent reflux of gastric contents. It is also important to understand the species differences when using an animal model to represent human physiology.

The aim of the present study was to determine which muscarinic receptors mediate contraction of porcine clasp and sling muscle fibers and to quantify the density of the total and of the individual M<sub>2</sub> and individual M<sub>3</sub> muscarinic receptor subtypes in these tissues. Results are compared to our previously reported findings in human clasp and sling fibers.
Methods:

Materials: All drugs and chemicals were obtained from Sigma Chemical Company (St. Louis, MO) except for darifenacin (which was a generous gift from Pfizer Limited, Sandwich, Kent), digitonin (Wako Pure Chemical Company, Osaka) and pansorbin (Calbiochem, La Jolla, CA).

Animal Specimens: Pig tissues were obtained from local slaughter houses. The pigs were 6-12 months of age and weighed approximately 125-170 lbs. Slaughter of the pigs followed rules and regulations written by the Food and Safety Inspection Service (FSIS), an agency of the United States Department of Agriculture (USDA).

Transport: Specimens were collected from each pig immediately post-slaughter. Each specimen included an entire stomach, part of the esophagus, and part of the crural diaphragm en bloc. The collected unit was temporarily preserved on ice and Tyrode’s solution (a modified Locke solution, used in physiological experiments, tissue cultures and tissue preservation). The ingredients in Tyrode’s solution (125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 23.8 mM NaHCO₃, and 5.6 mM glucose). The preserved specimens were transported to the laboratory within 1-2 hours of collection.

Dissection: Peritoneal fat was removed and dissection began using micro-scissors 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 towards the lesser curvature. The exact location of the sling and clasp fibers were identified at the greater and lesser curvature of GEJ, respectively, once the superficial longitudinal fibers were removed. Sling muscle fibers were removed from a

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relatively straight section of the greater curvature. Clasp fibers were obtained 1-2 cm
distal to GEJ along the lesser curvature. The muscles were further divided into
individual strips, each measuring 1-2 mm in width and 8-10 mm in length. Care was
taken to ensure the orientation of the muscle fibers parallel to the muscle strips and that
all strips were of uniform length and diameter. The muscle strips were then suspended
with 0.5 g of tension in tissue baths containing 10 ml of modified Tyrode's solution and
equilibrated with 95/5% O₂/CO₂ at 37°C.

Carbachol Concentration Response Curves: Following equilibration to the bath
solution for at least 30 minutes, the strips were challenged with 30 µM carbachol and
rinsed with buffer (5 exchanges over 60 minutes). This initial contraction to carbachol
was used to separate the strips into groups such that the average contraction of all
groups was the same. After washing, the strips were incubated for 30 minutes in the
presence or absence of one of 3 concentrations of the M₂ selective antagonist
methoctramine (0.1, 1 or 10 µM) or the M₃ selective antagonist darifenacin (30, 100, or
300 nM). Concentration response curves were derived from the peak tension
developed following the cumulative addition of carbachol (10 nM to 1 mM final bath
concentration). Either vehicle or one concentration of methoctramine or darifenacin was
used for each muscle strip. Because of tachyphylaxis in muscarinic receptor mediated
contraction, we could not repeat concentration response curves on the same muscle
strip, hence each strip could not be used as its own control for pairing EC₅₀ values.
Therefore, dose ratios were determined based on the average of the responses of
vehicle (H₂O) treated strips. This average EC₅₀ was used as a universal denominator to
determine the dose ratio for each strip in the presence of the antagonist. The data were
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normalized to the initial contraction induced by 30 µM carbachol. EC values were determined for each strip using a sigmoidal curve fit of the data (Origin, Originlab Corp. Northampton, MA.) and Schild plot's were constructed. If the 95% confidence interval of the slope of the Schild plot overlapped 1, the slope was constrained to 1 and the estimated pKb ± SEM is reported. If the 95% confidence interval of the slope of the Schild plot did not include 1, pA2 ± SEM is reported using the unconstrained slope.

Immunoprecipitation: Immunoprecipitation of muscarinic receptors from the individual dissections was performed as previously described (3). Briefly, the tissues were homogenized in cold Tris EDTA buffer (TE) with 10µg/mL of the following protease inhibitors: soybean and lima bean trypsin inhibitors, aprotinin, leupeptin, pepstatin, and α2-macroglobulin. 20µL of [³H] QNB (49 Curies/mM, approximately 4,000 cpm /µL) per mL assay homogenate was added and incubated at room temperature for 30 minutes with inversion every 5 minutes. Samples were pelleted via centrifugation at 20,000 g for 10 minutes at 4°C and the pellet was solubilized in TE buffer containing 1% digitonin and 0.2% cholic acid (1% TEDC) with the above protease inhibitors at 100 mg wet weight per ml. Samples were incubated for 50 minutes at 4°C, with inversion every 5 minutes then centrifuged at 30,000 g for 45 minutes 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, the supernatant containing the solubilized receptors bound with [³H] QNB were desalted over Sephadex G-50 minicolumns with 0.1% TEDC to separate the unincorporated ligand from the solubilized receptors. The amount of radioactivity in the eluate was determined by liquid scintillation spectrometry.
M₂ and M₃ receptors were precipitated by adding 200μL pansorbin, and incubated at 4°C for 50 minutes, with inversion every 5 minutes. The precipitated receptors were pelleted via centrifugation at 15,000 g for one minute at 4°C and the pellet was surface washed with 500μL of 0.1% TEDC. 50μL of 72.5mM deoxycholate/750mM NaOH was added and incubated for 30 minutes at room temperature. The pellet was resuspended in 1 mL of TE buffer and neutralized with 50μL of 1M 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 ± SEM) is reported as femtomoles (fMol) receptor per mg solubilized protein.
Results:

Immunoprecipitation: The results of the receptor density determinations are shown in table 1 as compared to our previous results in human tissue (4). As in the human tissue the total and M₃ receptor density in the pig sling fibers was statistically significantly greater than the clasp fibers. In contrast to the human data, the M₂ receptor density was slightly greater in the pig clasp than the sling fibers whereas M₂ receptor density is greater in human sling than clasp fibers. However, neither of these differences are statistically significant.

Concentration-effect relationships: Each muscle section was studied for isometric tension development in response to carbachol and each demonstrated a dose-related response to this agonist. Representative tracings for both clasp and sling fibers are show in figure 1. As seen in figure 2, the maximal carbachol induced contraction of clasp fibers is significantly less than sling fibers (p < 0.05), however, there is no difference in the potency of carbachol to mediate contraction between clasp and sling fibers. Figure 3 shows the graded concentration-effect relationship for carbachol in clasp (A, C) and sling fibers (B, D). Also shown in this figure are the curves for graded doses of this agonist with three different fixed concentrations of darifenacin, a relatively selective M₃ competitive antagonist and 3 different fixed concentrations of methoctramine, an M₂ selective antagonist. Schild plot’s for determining the potency of the antagonists to inhibit contraction are shown in figure 4. In clasp fibers, the intermediate potency of darifenacin (pKᵦ=8.0) suggests that both M₃ and M₂ receptors are involved in mediating contraction. Because of the low slope of the methoctramine Schild plot (0.22 ± 0.2, significantly less than 1), the methoctramine pA₂ value may be
unrelated to the disassociation constant at any one receptor subtype. The low slope
does, however, suggest that more than one receptor subtype may be involved in the
response. In the sling fibers, the relatively high potency of darifenacin (pKb=8.6)
suggest that M3 receptors mediate contraction. However, the low slope of the
methoctramine Schild plot (0.40 ± 0.1, significantly less than 1) may indicate that more
than one receptor subtype mediates contraction. These results, especially in the clasp
fibers, suggest that the carbachol induced contraction is mediated by both M2 and M3
receptors. Neither methoctramine nor darifenacin had significant effects on the maximal
contraction of the clasp fibers. However, both 0.1 and 10 µM methoctramine induced a
significant increase in contractile force in the sling fibers whereas neither 1 µM
methoctramine nor any concentration of darifenacin had any significant effects on the
maximal contraction in sling fibers. This augmentation of the contraction of sling fibers
by methoctramine suggests the possibility of inhibitory M2 receptors involved in
mediating contraction of the sling fibers.
Discussion:

The results of the current study demonstrate that the density of muscarinic receptor subtypes is different in the pig clasp and sling muscle fiber complex than in human clasp and sling muscle fiber complex (Table 1). However, the clasp and sling muscle fibers in both human and porcine, which work together to contract the GEJ and prevent reflux, have a greater density of M2 than of M3 receptors similar to most other smooth muscles studied.

The carbachol induced maximal contraction is greater in the pig sling muscle fibers than in the pig clasp muscle fibers. This result is in general agreement with a previous study showing that human sling muscle fibers contract significantly greater to acetylcholine than human clasp muscle fibers (22), and our prior study in human clasp and sling muscle fibers (4).

Classic 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. Schild analysis of our data yielded conflicting conclusions with respect to which receptor subtype mediates contraction of the gastric clasp and sling muscle fibers. In human sling fibers and both the human and porcine clasp muscle fibers, the M3 selective antagonist darifenacin yielded an inhibitory potency intermediate between that reported for M2 and M3 receptors, thus suggesting that both receptors may mediate the contractile response. In porcine sling fibers, darifenacin potency is high, consistent with M3 receptors mediating contraction. However, the Schild plot for the M2 selective antagonist methoctramine has a slope
significantly less than 1, which could indicate that more than one receptor subtype is involved in the contractile response.

The relatively high potency of darifenacin and the low slope of the methoctramine Schild plot in the pig sling fibers are consistent with a major M₃ receptor contribution and possibly a minor M₂ receptor contribution to mediating contraction in the sling fibers. However, in the clasp fibers, the relatively low potency of darifenacin and the low slope of the methoctramine Schild plot suggest that both M₂ and M₃ receptors mediate contraction. These results suggest that both M₂ and M₃ receptors mediate contraction in porcine clasp and sling fibers albeit likely with differences in the relative contribution of each receptor subtype in each tissue. Based on the result that darifenacin is less potent in inhibiting clasp fibers contraction than sling fiber contraction, we conclude that the M₂ receptor has a greater contractile role in clasp fibers than in sling fibers. The lower potency of darifenacin to inhibit contraction of the porcine clasp fibers than sling fibers may be related to the lower density of M₃ receptors in the clasp fibers than in the sling fibers.

The M₂ selective antagonist, methoctramine significantly augmented the Emax response to carbachol in sling muscle fibers. This finding suggests the possibility of inhibitory M₂ receptors in porcine sling muscle. When these inhibitory receptors are blocked with methoctramine, the contraction is augmented. Another possible explanation is that the signal transduction mechanisms activated by M₂ receptors inhibits the signaling from M₃ receptors in the muscle. This would result in a sub-additive interaction. An increased contractile response could occur as a consequence of blocking the M₂ receptor inhibition on M₃ signaling by methoctramine.
Due to the lack of completely specific antagonists, the contribution of the individual receptor subtypes cannot be precisely determined in either pig or human tissue. Because it has been established that both M2 and M3 muscarinic receptors contribute to the carbachol mediated contraction in the gastric sling and clasp muscle fibers there is interest in determining whether these two receptor subtypes interact. Toward that end we have previously developed the theoretical framework and associated experimental procedure that quantitates that interaction. That methodology, however, requires occupation-effect data for each receptor type when it is the sole receptor producing the effect. That was achieved, and the method applied, in our earlier publication (5) which utilized knockout (KO) mice. In that study isolated strips from the M2-KO and the M3-KO each gave dose-related effects that allowed prediction of the dose-effect relation accompanying the dual occupancy (the usual, wild type case). That curve of prediction, when compared with corresponding experimental data, leads to a measure of the interaction. In that mouse experiment, the interaction was simply additive, but the methodology sets the stage for examination of such interactions in the human and porcine preparations discussed here. For these species, however, we lack the needed knockouts and, therefore, efforts are under way to employ highly selective M2 competitive antagonists (9) to yield the needed single receptor occupation-effect data. The current paper’s results (receptor density and K values) provide a guide for finding the appropriate antagonist doses that might achieve this objective and the results of that study will be the subject of a future communication.

In summary, the receptor density of each smooth muscle group differs according to the muscle location, function, and species. It was found that, similar to human clasp
and sling muscle fibers, porcine clasp and sling muscle fiber contraction is mediated by both M₂ and M₃ receptors.
Acknowledgments:
The authors would like to acknowledge the expert technical assistance of Elan S. Miller, Gabrielle N. Soussan and Imran Hamid in carrying out the contractility studies.

Grants:
This work was funded by National Institutes of Health grant [RO1DK059500].
References:


Legends for Figures:

Figure 1. Original tracings of carbachol concentration response experiments from pig clasp and sling muscle fibers.

Figure 2. Carbachol concentration response curves for porcine clasp and sling fibers. * denotes statistically significant difference in maximal contraction between clasp and sling fibers (p < 0.05, Mann-Whitney U test), n= number of muscle strips, N= number of pigs.

Figure 3. Concentration response curves for carbachol induced contraction of clasp (A, C) and sling fibers (B, D) in the presence of various concentrations of methoctramine (A, B) and darifenacin (C, D). Results are shown as percent of the initial contractile response induced by 30 µM carbachol. n= number of muscle strips. All results are pooled from 4 different animals. * denotes statistically significant difference in maximal contraction from vehicle (p < 0.05, Student’s t test).

Figure 4. Schild plots for determination of the potency of methoctramine (A, B) and darifenacin (C, D) for inhibition of carbachol induced contraction of porcine clasp (A, C) and sling (B, D) fibers. * denotes that the slope of the Schild plot is significantly less than unity.
Table 1. Comparison of M₂ and M₃ muscarinic receptor density in pig and human clasp and sling fibers.

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<tr>
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<th>Pig</th>
<th>Human</th>
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<tr>
<td></td>
<td>Total</td>
<td>M₂</td>
</tr>
<tr>
<td>Clasp</td>
<td>797±14</td>
<td>512±17</td>
</tr>
<tr>
<td>Sling</td>
<td>856±27*</td>
<td>443±39</td>
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Receptor density is expressed as fmol receptors per mg solubilized protein. Values for human tissues was extracted from our previously published results (4). * denotes significant difference (P < 0.05, Student’s t test) between clasp and sling fibers.
Figure 1

[Carbachol]
Porcine Gastroesophageal Muscle Fibers

Clasp n=41, N=13

Sling n=44, N=16

Tension (g) vs. Log[Carbachol]

Figure 2
A. Clasp

B. Sling

C. Clasp

D. Sling

Figure 3
A. Clasp
\[ pA_2 = 7.9 \pm 0.6 \]
\[ \text{slope} = 0.22 \pm 0.2^* \]

B. Sling
\[ pA_2 = 7.7 \pm 0.6 \]
\[ \text{slope} = 0.40 \pm 0.1^* \]

C. Clasp
\[ p_{kb} = 8.0 \pm 0.2 \]
\[ \text{slope} = 0.97 \pm 0.5 \]

D. Sling
\[ p_{kb} = 8.6 \pm 0.3 \]
\[ \text{slope} = 0.85 \pm 0.5 \]

Figure 4