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Nicotinic Receptor Subtypes Mediating Relaxation of the Normal Human Clasp and Sling Fibers of the Upper Gastric Sphincter

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Abstract

Background—Proper function of the gastroesophageal high pressure zone is essential for the integrity of the antireflux barrier. Mechanisms include tonic contractions as well as the decreased tone during transient lower esophageal sphincter relaxations.

Methods—We characterized the pharmacology of nicotinic receptors mediating relaxations of the human upper gastric sphincter (clasp and sling fibers) using currently available subtype selective nicotinic antagonists in tissue from organ transplant donors. Donors with either a history of gastroesophageal reflux disease or histologic evidence of Barrett's esophagus were excluded. Clasp and sling muscle fiber strips were used for one of three paradigms. For paradigm 1, each strip was exposed to carbachol, washed, exposed to nicotinic antagonists then re-exposed to carbachol. In paradigm 2, strips were exposed to a near maximally effective bethanechol concentration then nicotine was added. Strips then were washed, exposed to nicotinic antagonists then re-exposed to bethanechol followed by nicotine. In paradigm 3, strips were exposed to bethanechol then choline or cytosine.

Key Results—100 μ M methyllycaconitine has no inhibitory effects on relaxations, eliminating homomeric $\alpha 7$ subtypes. Subtypes composed of $\alpha 4\beta 2$ subunits are also eliminated because choline acts as an agonist and dihydro-beta-erythroidine is ineffective.

Conclusions & Inferences—Because mecamylamine blocks the relaxations and both choline and cytosine act as agonists in both clasp and sling fibers, the nicotinic receptor subtypes responsible for these relaxations could be composed of $\alpha 3\beta 4\beta 2$, $\alpha 2\beta 4$ or $\alpha 4\beta 4$ subunits.

Keywords

gastric clasp fibers; gastric sling fibers; smooth muscle; nicotinic receptors; enteric nerves; gastroesophageal reflux

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Introduction

Gastroesophageal reflux disease (GERD) affects at least 20% of the population (6) and represents a huge healthcare burden. Although effective symptomatic acid suppressive treatments for GERD are available, they do not prevent reflux of gastric contents. GERD is associated with serious sequela ranging from the minor inconveniences of heartburn to the lethal complications of aspirational pneumonia in infancy and esophageal adenocarcinoma in adulthood. Finding treatment strategies that prevent GERD is a high public health priority.

Proper function of the mechanisms of neuromuscular control of the gastroesophageal high pressure zone (HPZ) is likely to be essential for the integrity of the antireflux barrier. These mechanisms include those responsible for the tonic contractions as well as the decreased tone during transient lower esophageal sphincteric relaxations (TLESR). It has been known for over five decades that in human subjects, administration of the muscarinic receptor antagonist drug atropine reduces the intraluminal pressure of the HPZ (2, 3, 10, 11). This indicates that a portion of the in-vivo tonic muscle tone of the sphincteric mechanisms in the HPZ is maintained by the neuronal release of acetylcholine acting on smooth muscle muscarinic receptors. The anatomic structures of the HPZ responsible for reflux prevention include the lower esophageal circular smooth muscle (LEC, often referred to as the lower esophageal sphincter, LES), the crural diaphragm skeletal muscle, the clasp and sling smooth muscle fibers of the proximal stomach (which we refer to as the upper gastric sphincter, UGS) and very likely, the muscularis mucosa smooth muscle. We previously demonstrated that in patients with GERD, the UGS does not contribute to intraluminal pressure of the HPZ as it does in normal volunteers (9) suggesting that the underlying etiology of GERD may be a pathophysiologic defect within the gastric clasp/sling smooth muscle fiber complex.

The clasp and sling fibers of the upper gastric sphincter are mechanically arranged such that the U shaped sling fibers loop around the esophageal opening at the greater curvature of the stomach with the open ends extending on both sides of the esophageal opening towards the lesser curvature of the stomach and these are connected with each other by the clasp fibers (8). When clasp and sling fibers contract in concert, this arrangement acts like a necktie, constricting esophageal opening. This is one of the first barriers that the contents of the stomach encounter before reflux up the esophagus occurs. If the clasp fibers cannot maintain a close approximation between the open ends of the U shaped sling fibers then no matter how much the sling fibers contract, the esophageal opening will not be constricted. Likewise if the sling fibers cannot contract then no matter how closely opposed the open ends are maintained by contraction of the clasp fibers, constriction of the esophageal opening will not occur because the sling fibers looping around the greater curvature will not be pulled tight around the esophageal opening. Thus muscarinic receptor mediated contractions and nicotinic receptor mediated relaxations in these two groups of fibers are likely to be important for the proper function of the UGS in prevention of esophageal reflux.

In order to understand pathophysiologic neuromuscular mechanisms underlying the UGS defect in GERD, it is first necessary to establish the normal mechanisms in human tissues from subjects without GERD. Because detailed pharmacologic studies of contractile responses are not possible using the minute tissue obtainable with an endoscopic biopsy, we have been studying contractile responses in smooth muscle strips from whole stomach and esophagus specimens obtained from organ transplant donors. Because atropine reduces intraluminal HPZ pressure in-vivo, we have been using relaxation of muscarinic receptor pre-contracted clasp and sling muscle as an in vitro physiologic equivalent of in vivo TLESR. We previously found that nicotine causes relaxation of bethanechol pre-contracted clasp, sling and LEC muscle strips and that this effect occurs indirectly, by activation of nicotinic receptors on enteric nerves that induces release of multiple relaxing substances. These relaxing substances include nitric oxide (NO) in clasp, sling, and LEC fibers, norepinephrine acting on β -adrenoceptors in clasp fibers, and GABA acting on GABA_A receptors in sling fibers (5). The current study was undertaken to characterize the pharmacology of the enteric nerve nicotinic receptors mediating these relaxations of the UGS using currently available nicotinic receptor subtype selective agents.

Materials and Methods

These studies were performed using 14 human stomachs and esophagi obtained over 42 months from organ transplant donors procured through the National Disease Research Institute (Philadelphia, PA) or the International Institute for the Advancement of Medicine (Edison, NJ). These specimens were from brain dead organ transplant donors maintained on life support whose next of kin consented to donation of organs for research. The specimens were harvested within 30 minutes after cross clamping the aorta and the stomach contents were rinsed out with saline. The esophageal and pyloric openings were ligated and the tissue was placed in either University of Wisconsin (Beltzer's Viaspan) organ transport media or HTK (composed of, in mM: NaCl-15, KCl-9, Potassium hydrogen 2-ketoglutarate-1, MgCl₂-4, histidine NaCl-18, tryptophan-2, mannitol-30, CaCl₂-0.015). The specimens were transported to the laboratory by overnight courier on ice.

The only medical records available relate to the events occurring at the time of brain death because the donors' identity was de-identified by the procurement agencies. Thus limited medical history is available and no direct medical record information is accessible to determine whether the subject had GERD diagnosed by a physician. Indirect medical history was obtained by the procurement agencies by interviewing the next of kin and determining whether the donor had heartburn, reflux, regurgitation, prior endoscopy for GERD, documented GERD on endoscopy, biopsy evidence of GERD, use of antacids, proton pump inhibitors or histamine H₂ receptor blockers on a regular basis, a diagnosis of Barrett's esophagus, or prior surgery for GERD.

For each specimen, the transition zone was fixed in formalin, stained with Alcian blue and examined for the presence of goblet cells in the gastric mucosa for the histologic diagnosis of Barrett's esophagitis (Barrett's) as previously described (7). Specimens were determined to be from non-GERD donors if they had no indirect medical history of GERD and no goblet cells in the gastric mucosa. All donors with either a history of GERD or a histologic

diagnosis of Barrett's esophagitis were excluded from these studies. These studies were performed on non-GERD specimens only.

The specimens were dissected in a cold room (0–5°C). The greater and lesser omenta were removed and the outermost longitudinal fibers descending from the esophagus across the stomach were removed by sharp dissection to expose the sling and clasp muscle fibers. The sling muscle fibers are a U shaped group of fibers surrounding the esophagus around the greater curvature of the stomach while the clasp muscle fibers are a semicircular group of muscle fibers located on the lesser curvature side of the stomach opposite to the cardiac notch. The clasp muscle fibers are oriented perpendicular to the sling muscle fibers between the open ends of the U shaped sling muscle fibers. The clasp and sling muscle fiber complexes were separated from the underlying submucosa then the clasp fibers were removed from the sling fibers by sharp dissection. Clasp fibers were cut into 24–32 muscle strips approximately $3 \times 3 \times 8$ mm with the long axis parallel to the direction of the muscle fibers. Similar sized muscle strips were cut from the sling fiber complex such that these strips were derived from the sling muscle fibers in the cardiac notch as well as fibers extending along both sides of the esophageal opening of the stomach. The muscle strips were suspended between force transducers and positioners in muscle baths with 10 ml Tyrode's solution continuously bubbled with 95% O₂/5% CO₂ and maintained at 37°C. The strips were stretched to approximately 150% of their slack length which produced approximately 1 gram of basal tension and were then allowed to accommodate to the muscle bath for at least 60 minutes.

Paradigm 1

Responses to the mixed muscarinic and nicotinic receptor agonist carbachol were measured following cumulative additions in half log concentration intervals from 30 μ M (3E-5 M) to 1 mM (1E-3 M). Responses were allowed to develop for 2–4 minutes before the addition of the next higher concentration of carbachol. Because some subtypes of nicotinic receptors are rapidly desensitized, complete concentration response curves (CRC) were not performed on all strips. The muscle strips were washed by exchange of bathing solution at 10 min intervals over 60 minutes prior to the addition of either antagonist or vehicle and equilibrated for 30 min before repeating the cumulative carbachol CRC. Thus each muscle strip was exposed to carbachol 2 times. The second responses to carbachol, after the addition of either nicotinic receptor antagonists or vehicle, were compared to the first responses and vehicle treated strips. See figure 1 for representative tracings of the various experimental paradigms used in the current study. The antagonists used were antagonists of either ganglionic nicotinic receptors, mecamylamine (10 μ M) and hexamethonium (100 μ M) or the neuromuscular nicotinic receptor antagonist decamethonium (300 μ M). These concentrations were chosen to maximally inhibit nicotinic receptors. Because of limitations in the number of strips obtainable from these specimens, additional antagonist concentrations could not be tested. Strips were used in one of 3 different experimental paradigms as illustrated in figure 1.

Paradigm 2

Strips were exposed to a near maximally effective concentration of bethanechol (30 μ M) then, after reaching maximal tension, 1 mM nicotine was added. In order to minimize any possible effects of nicotine induced receptor desensitization, only a single concentration of nicotine was used. After 60 minutes of repeated washing (exchange of buffer every 10 min) and re-equilibration, strips were exposed to either vehicle or nicotinic receptor antagonists for 30 minutes then re-challenged with bethanechol followed by nicotine. The antagonists studied were methyllycaconitine (MLA, 100 nM), mecamylamine (10 μ M), hexamethonium (100 μ M), decamethonium (300 μ M) and dihydro- β -erythroidine (10 μ M). These antagonist concentrations were chosen based on previously reported potencies for nicotinic receptors (1).

Paradigm 3

A third experimental paradigm was used to determine if choline or cytosine are agonists for the nicotinic receptor subtype which induces relaxation of the clasp and sling muscle fibers. The muscle strips were also exposed to 30 μ M bethanechol, however, instead of adding nicotine, either vehicle, choline (1 μ M to 10 mM, log fold increments) or cytosine (1 μ M to 1 mM, log fold increments) were added cumulatively every 3 minutes. At the conclusion of the concentration response curves, 1 mM nicotine was added to all muscle strips.

Data is shown as mean $\pm$ SEM for the number of muscle strips (n) from the number of donor organs (N). Tension developed by the muscle fibers is not normalized to cross sectional area because analysis of over 300 clasp and 300 sling fibers demonstrated that there is no significant correlation between the maximal response to bethanechol and the cross sectional area of either clasp or sling muscle strips. This may be due to the manner in which the muscle strips are suspended in the tissue baths. The strips are attached via tissue clips (Radnoti LLC, Monrovia, CA, cat # 158801) which meet at a point and do not encompass the entire cross sectional area of the strip. Hence the contraction measured may be due to only a linear portion of the muscle strip located between the clips. Statistical analyses were performed using analysis of variance. Because the variance was significantly different between groups, a nonparametric Mann-Whitney U-test was used to compare groups. Probability values less than 0.05 are considered statistically significant.

Results

Patient Demographics

The donors consisted of 8 males and 6 females. Nine were Caucasian, 2 were Asian, 1 was Indian, 1 was Hispanic and 1 was African American. The average age was 43.7 ± 4.2 years, the average height was 1.7 ± 0.03 meters, the average weight was 80.9 ± 6.1 kg and the average body mass index was 28.3 ± 2.1 .

Responses to Carbachol

Preliminary experiments using complete carbachol concentration response curves (1E-8 to 1E-3 M, half log intervals) demonstrate that carbachol concentrations greater than 1E-4 M induced relaxations in both clasp and sling fibers (Figure 3). In order to minimize the

possible effects of carbachol induced desensitization of nicotinic receptors, an abbreviated carbachol CRC was performed to determine the effects of the nicotinic receptor antagonists. Clasp and sling muscle strips contracted when exposed to 30 μ M carbachol. The next higher concentration of carbachol (100 μ M) induced a slight contraction in some strips and a slight relaxation in other strips. The highest 2 concentrations of carbachol (300 μ M and 1 mM) induced rapid relaxations in both clasp and sling muscle fibers from all donor tissues. Representative traces of these responses in clasp fibers are shown in the left side panels of figure 2. As can be seen in figure 4A, sling fibers contracted significantly more than clasp fibers (open bars) while clasp fibers relaxed significantly more than sling fibers (shaded bars) to carbachol in terms of grams. When the relaxation was normalized to maximal tension, clasp fibers relaxed significantly more than sling fibers to carbachol as a percentage of the contractile response (Figure 4B, open bars).

Responses to Bethanechol and Nicotine

All clasp and sling fibers contracted in response to 30 μ M bethanechol and relaxed to 1 mM nicotine. Representative traces of these responses for clasp fibers are shown in the right side panels of figure 2. As can be seen in figure 4A, similar to the responses to carbachol, the contractile response to bethanechol was significantly greater in sling fibers than clasp fibers (hatched open bars). However, sling fibers relaxed significantly more than clasp fibers to 1 mM nicotine in terms of grams (Figure 4A, shaded hatched bars). Because sling fibers contracted more than clasp fibers, when the relaxation is normalized to maximal tension, sling fibers relaxed significantly less than clasp fibers to nicotine (Figure 4B, shaded bars).

Comparison of the Carbachol Responses to Bethanechol and Nicotine Responses

Clasp fibers contracted significantly more to carbachol than to bethanechol, whereas the relaxation response to carbachol was not different than the relaxation response to nicotine in terms of grams (Figure 4A). However, when normalized to maximal tension, clasp fibers relaxed significantly more to nicotine than to carbachol (Figure 4B). In sling fibers, the contractile response to bethanechol and carbachol was not statistically significantly different but the relaxation response was significantly greater to nicotine than to carbachol in terms of grams of tension (Figure 4A) or as a percentage of maximal tension developed (Figure 4B).

Comparison of the First and Second Carbachol Concentration Response Curves (CRC)

In this paradigm, a carbachol CRC was performed on all muscle strips. After washing, some muscle strips were incubated with vehicle while others were incubated with nicotinic receptor antagonists. After this incubation, a second carbachol CRC was performed on all muscle strips (Figure 2, left side panels). Clasp fibers contracted significantly less (Figure 5A, VEH bars) and relaxed the same (Figure 5C, VEH bars) to the second carbachol CRC as compared to the first carbachol CRC. In contrast, sling fibers contracted the same (Figure 5B, VEH bars), but relaxed significantly more (Figure 5D, VEH bars) to the second carbachol CRC.

Antagonist Effects on Carbachol Contraction and Relaxation

None of the antagonists had a significant effect on the magnitude of the carbachol induced contractile response in either clasp or sling fibers compared to vehicle (Figure 5A and 5B). In clasp fibers, decamethonium and mecamlamine inhibited carbachol induced relaxation, while hexamethonium had no effect (Figure 5C). In sling fibers, decamethonium, mecamlamine and hexamethonium all inhibited carbachol induced relaxation (Figure 5D). In clasp fibers, none of the antagonists had a significant effect on the potency of carbachol for inducing relaxation, however, decamethonium and mecamlamine reduced the maximal relaxation to carbachol (Table 1). In sling fibers, decamethonium decreased the potency of carbachol for inducing relaxation, while decamethonium, mecamlamine and hexamethonium reduced the maximal relaxation in response to carbachol.

Comparison of the First and Second Responses to Bethanechol and Nicotine

In this experimental paradigm, all muscle strips were stimulated to contract with 30 μ M bethanechol then induced to relax with 1mM nicotine. After washing, some muscle strips were incubated with vehicle while others were incubated with nicotinic receptor antagonists. All strips were exposed to bethanechol and nicotine a second time (Figure 2, right side panels). There was no difference in the contraction of clasp fibers between the two exposures to bethanechol (figure 6A, VEH bars). However, clasp fibers relaxed significantly more to the second exposure of nicotine (Figure 6C, VEH bars). In sling fibers, there was no difference between either the contraction to bethanechol or the relaxation to nicotine between the first and second exposures (Figure 6B and 6D, VEH bars).

Antagonist Effects on Bethanechol Contraction and Nicotine Relaxation

None of the antagonists had a significant effect on the magnitude of the bethanechol induced contractile response in either clasp or sling fibers compared to vehicle (Figure 6A and 6B). MLA had no effect, while mecamlamine, hexamethonium and decamethonium inhibited nicotine induced relaxations in both clasp and sling fibers (Figure 6C and 6D). 10 μ M dihydro- β -erythroidine also had no effect on the contractile response to bethanechol or nicotine induced relaxations in either clasp or sling fibers (n=8 clasp strips from 2 donors, n=7 sling fibers from 2 donors, data not shown).

Relaxation Responses of Choline and Cytisine

In this paradigm all muscle strips were exposed to 30 μ M bethanechol to induce contraction, followed by the addition of vehicle, choline or cytisine to determine if these agents act as agonists for relaxation. Representative traces of these responses for clasp fibers are shown in the bottom left panels of Figure 2. As can be seen in figures 7A and 7B, 10 mM choline induced a significant relaxation as compared to vehicle in both clasp and sling fibers. The choline induced relaxation was statistically significantly less than the relaxation induced by nicotine in clasp fibers. In sling fibers, there was no statistically significant difference between the choline and nicotine induced relaxations. Likewise cytisine at 100 μ M induced a maximal relaxation in both clasp and sling fibers that was not different than the nicotine induced relaxations (n=4 clasp and n=4 sling strips from 1 donor, data not shown). The relaxation response to nicotine in the presence of choline was not significantly different than

the relaxation to nicotine alone. Thus choline does not act as an antagonist at the nicotinic receptors mediating relaxation in either clasp or sling fibers.

Discussion

There are no other experiments reported in the existing literature that determine the subunit composition of the nicotinic receptor subtypes that mediate relaxation of the clasp and sling muscle fibers in the UGS. Isolating individual specific nicotinic receptor subunits that are responsible for these relaxations could lead to therapeutic targets to prevent GERD.

Both carbachol and bethanechol caused greater contractions in sling than clasp fibers, consistent with previous reports by us (4, 5) and others (12). The greater contractions induced in sling fibers may be a consequence of our previously reported findings of nearly a two-fold higher total muscarinic receptor density and over 7 fold higher M₃ muscarinic receptor subtype density in sling versus clasp fibers (4). The relaxant response to higher carbachol concentrations is greater in clasp than sling fibers when expressed as a percentage of the maximal contractile response (figure 4B). Likewise we found that the relaxant response to nicotine after inducing a maximal contractile response with bethanechol is also greater in clasp than sling fibers as we previously reported (5). Both clasp and sling fibers relax more to nicotine following bethanechol induced contractions than to higher concentrations of carbachol, which is consistent with desensitization of the relaxing nicotinic receptors during the contractile phase of the response to the cumulative additions of carbachol. However, carbachol and bethanechol produce the same degree of contraction in sling fibers but carbachol causes a greater contraction than bethanechol in clasp fibers.

We previously reported that without pre-contracting the strips to bethanechol, sling fibers contract but clasp fibers relax in response to 1 mM nicotine (5). In sling fibers, it seems that the nicotinic receptor induced contractile effects of carbachol do not combine with the contractile effects of carbachol mediated by muscarinic receptors. On the other hand, in clasp fibers, the nicotinic and muscarinic receptor stimulating effects of carbachol combine to induce a greater contraction than selectively stimulating only muscarinic receptors with bethanechol despite our finding that nicotine induces relaxation of clasp fibers that are not pre-contracted by muscarinic receptor stimulation with bethanechol. It is possible that during the contractile response to carbachol in clasp fibers, the nicotinic receptor stimulation induces release of contractile transmitters causing effects that combine with the muscarinic receptor stimulating effects of carbachol augmenting the contraction induced by muscarinic receptor stimulation alone. Thus sling fibers have a greater propensity to contract in response to cholinergic stimulation mediated by muscarinic receptors. Conversely, clasp fibers have a greater propensity to relax to cholinergic stimulation mediated by nicotinic receptors. This raises the question of whether differences in nicotinic receptors in clasp versus sling fibers may underlie this difference in nicotinic receptor mediated relaxation of muscarinic receptor pre-contracted fibers.

Nicotinic receptors are ligand gated cation channel receptors composed of a pentameric arrangement of α and β subunits including the γ subunit in immature and the ϵ subunit in mature skeletal muscle. In neuronal tissue, at least 8 different types of α subunits ($\alpha 2$

through $\alpha 9$) and 3 types of β subunits ($\beta 2$ through $\beta 4$) are found. Because of this subunit diversity, the subunit compositions, stoichiometries and subunit arrangements of naturally expressed nicotinic receptor subtypes are not known with complete certainty. Not all possible combinations of α and β subunits lead to functional ligand gated cation channel receptors. However, the potential large diversity in subunit composition greatly exceeds the capacity of selective ligands to definitively distinguish individual subtypes based on the selectivity of the ligands. We determined the subunit composition of the nicotinic receptors mediating relaxation of the clasp and sling muscle fibers using the more general approach of investigating nicotinic receptor agonists and antagonists that affect broad classes of nicotinic receptors producing different current types.

Based on comparison of drug effects on electrophysiological events in rodent neuronal cells in either brain slices or primary cell culture with electrophysiological events in cellular systems transfected with various permutations of genes encoding the individual subunits, nicotinic receptors have been classified into 4 different current types. Type 1A currents, produced in cells transfected with only the $\alpha 7$ subunit (homomeric $\alpha 7$ receptors), are inhibited over 95% by 10 nM MLA, less than 10% by 1 μ M mecamylamine and both choline and cytosine are full agonists. For type II currents, produced in cells transfected with both $\alpha 4$ and $\beta 2$ subunits, 10 μ M dihydro-beta-erythroidine blocks over 95% of currents, MLA does not inhibit and choline is not an agonist but cytosine is a partial agonist. Cells transfected with $\alpha 3$, $\beta 4$ and $\beta 2$ subunits produce type III currents which are not inhibited by MLA but 1 μ M mecamylamine blocks 80% of the current, choline acts as a partial agonist and cytosine is a full agonist. A type IV current is also described in cells transfected with $\alpha 2$ and $\beta 4$ or $\alpha 4$ and $\beta 4$ subunits and this current is not inhibited by MLA and cytosine is a full agonist. See (1) for review. Homomeric $\alpha 7$ nicotinic receptor subtypes (producing Type 1A currents) are not responsible for the nicotine induced relaxations in either clasp or sling fibers because 100 μ M MLA had no inhibitory effects on relaxations in clasp or sling fibers (Figure 6). Because choline acts as an agonist in both clasp and sling fibers and dihydro-beta-erythroidine is ineffective, receptor subtypes composed of $\alpha 4\beta 2$ subunits (producing Type II currents) are also not responsible for these relaxations. Because mecamylamine blocks the relaxations and both choline and cytosine act as agonists in both clasp and sling fibers, the nicotinic receptor subtypes responsible for these relaxations could be composed of either $\alpha 3\beta 4\beta 2$ subunits (producing Type III currents) or $\alpha 2\beta 4/\alpha 4\beta 4$ subunits (producing Type IV currents).

Besides the differences between clasp and sling fibers in contractile response to muscarinic receptor stimulation and the subsequent relaxant response to nicotinic receptor stimulation, one other major difference between these two fiber types was uncovered by this investigation. Although the neuronal nicotinic receptor blocking agent hexamethonium does prevent the nicotine induced relaxation in bethanechol pre-contracted clasp and sling fibers and the relaxation to higher concentrations of carbachol in sling fibers, hexamethonium is ineffective in preventing the relaxations induced by higher carbachol concentrations in clasp fibers. Thus the nicotinic receptors mediating relaxations are slightly different between the clasp and sling fibers of the UGS. If these nicotinic receptors in clasp and sling fibers are different than the ganglionic nicotinic receptors found elsewhere that mediate major

functions such as blood pressure, then selective inhibition of the unique nicotinic receptors in the UGS may be an effective treatment for GERD.

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Glossary of Abbreviations

GABA	gamma amino butyric acid
GERD	Gastroesophageal reflux disease
HPZ	high-pressure zone
GTK	histidine, tryptophan, ketoglutarate media
LEC	lower esophageal circular muscle
LES	lower esophageal sphincter
MLA	methyllcaconitine
TLESR	transient lower esophageal sphincter relaxation
UGS	upper gastric sphincter
VEH	vehicle

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Key Message

The nicotinic receptors responsible for relaxation of the gastric clasp and sling fibers that compose the upper gastric sphincter were investigated in 14 whole human stomach and esophagus specimens obtained from organ transplant donors using currently available nicotinic receptor subtype selective agonists and antagonists. Because mecamylamine blocks the relaxations and both choline and cytosine act as agonists in both clasp and sling fibers, the nicotinic receptor subtypes responsible for these relaxations could be composed of either $\alpha 3\beta 4\beta 2$ subunits (producing Type III currents) or $\alpha 2\beta 4/\alpha 4\beta 4$ subunits (producing Type IV currents). If these nicotinic receptors in clasp and sling fibers are different than the ganglionic nicotinic receptors found elsewhere that mediate major functions such as blood pressure, then selective inhibition of the unique nicotinic receptors in the UGS may be an effective treatment for GERD.

Figure 1.

Diagram illustrating the 3 different experimental paradigms used to investigate nicotinic receptor mediated relaxation of smooth muscle fibers of the UGS. For paradigm 1 carbachol is used in a cumulative fashion causing contraction by action on smooth muscle muscarinic receptors then, at higher concentrations, causing relaxations by action at nicotinic receptors on inhibitory enteric neurons. After washing and adding nicotinic receptor antagonists, the cumulative carbachol exposure is repeated. In Paradigm 2 bethanechol is first used to induce smooth muscle contraction by action on smooth muscle muscarinic receptors then nicotine is

added to activate the inhibitory enteric neurons. After washing and adding nicotinic receptor antagonists, the bethanechol followed by nicotine exposure is repeated. For paradigm 3, bethanechol is added to induce smooth muscle contraction then choline or cytosine are added in a cumulative fashion to induce relaxation by activation of nicotinic receptors on the inhibitory enteric neurons.

Paradigm 1

Paradigm 2

Paradigm 3

Figure 2.

Representative traces for clasp fibers demonstrating the different experimental paradigms. The time and tension indicators are shown at the bottom left. Contractile responses were determined by the differences in tension immediately before adding the drug dose to the bath and the peak response within 2–4 minutes after drug exposure. Relaxant responses were determined by the difference between the maximal contractile response and the minimal tension within 2–4 minutes after adding the relaxatory substances. Drug concentrations are shown in molar using engineering notation such that E refers to base 10 exponent.

Figure 3.

Carbachol concentration response curves in clasp and sling fibers. Human clasp and sling fibers were exposed to the cumulative addition of carbachol at half log intervals from 10 nM to 1 mM. Responses are shown as the mean $\pm$ SEM as the percentage of the maximal contraction to carbachol. For clasp fibers, n=34 strips from 4 donors and for sling fibers, n=31 strips from the same 4 donors.

Figure 4.

A. Contraction and relaxation of clasp and sling fibers in response to carbachol and bethanechol followed by nicotine. Human clasp and sling fibers were exposed to either cumulative concentrations of carbachol at half log intervals from 30 μ M to 1 mM or were pre-contracted with 30 μ M bethanechol and induced to relax w 1 mM nicotine. The contraction and relaxation (maximal) responses are shown in grams of tension and represent the mean $\pm$ SEM. For the carbachol studies, n=53 strips from 6 donors for clasp and n=39 strips from 3 donors for sling. For the bethanechol followed by nicotine studies, n=61 strips

from 8 donors for clasp and n=71 strips from 8 donors for sling. ** denotes significant difference of $p<0.01$. B. Relaxation of clasp and sling fibers in response to carbachol and nicotine. The relaxation data from panel A is shown as a percentage of the maximal tension and represent the mean $\pm$ SEM. ** denotes significant difference of $p<0.01$.

Figure 5.

Effect of decamethonium (DECA), mecamlamine (MECA) and hexamethonium (HEX) on the contraction and relaxation responses of clasp and sling muscle fibers in response to carbachol. A cumulative carbachol CRC was performed on clasp (panels A and C) and sling (panels B and D). The maximal contraction (panels A and B) and maximal relaxation response are shown as mean $\pm$ SEM for the first CRC (Pre) and the second CRC after the addition of either vehicle or antagonist (Post). For clasp fibers, n=27 strips from 6 donors, n=8 strips from 1 donor, n=6 strips from 2 donors and n=12 strips from 3 donors for vehicle

(VEH), DECA, MECA and HEX respectively. For sling fibers, n=15 strips from 3 donors, n=4 strips from 1 donor, n=12 strips from 2 donors and n=8 strips from 2 donors for VEH, DECA, MECA and HEX respectively. ## denotes significant difference of $p < 0.01$ between the first response and the second response (vehicle only). * denotes significant difference $p < 0.05$ between antagonist treated and vehicle treated groups.

Figure 6.

Effect of methyllycaconitine (MLA), decamethonium (DECA), mecamlamine (MECA) and hexamethonium (HEX) on bethanechol induced contraction and nicotine induced relaxation of clasp and sling muscle fibers. Clasp (panels A and C) and sling (panels B and D) muscle fibers were first (Pre) pre-contracted with 30 μ M bethanechol (panels A and B) and induced to relax with 1 mM nicotine (panels C and D). Following washing and incubation with antagonists, the strips were treated with bethanechol and nicotine a second time (Post). Data is shown as mean $\pm$ SEM. For clasp fibers, n=31 strips from 7 donors,

n=17 strips from 5 donors, n=2 strips from 1 donor, n=13 strips from 4 donors and n=5 strips from 2 donors for VEH, MLA, DECA, MECA and HEX respectively. For sling fibers, n=33 strips from 7 donors, n=21 strips from 5 donors, n=5 strips from 1 donor, n=17 strips from 4 donors and n=7 strips from 2 donors for VEH, MLA, DECA, MECA and HEX respectively. # denotes significant difference of $p<0.05$ between the first response and the second response (vehicle only). ** denotes significant difference $p<0.01$ between antagonist treated and vehicle treated groups.

Figure 7.

Choline CRC for relaxation in clasp and sling muscle fibers. Clasp (panel A) and sling (panel B) muscle fibers were pre-contracted with 30 μ M bethanechol and induced to relax with increasing concentrations of choline or vehicle. Following the last application of choline or vehicle 1mM nicotine was added to all strips. Data is shown as mean $\pm$ SEM. For clasp fibers, n=10 strips from 3 donors for vehicle and n=8 strips from 3 donors for choline. For sling fibers, n=10 strips from 3 donors for vehicle and n=8 strips from 3 donors for choline. ** denotes significant difference $p < 0.01$ between the choline treated group and the

vehicle treated group. ## denotes significant difference of $p < 0.01$ between the maximal choline induced relaxation and the nicotine induced relaxation in the vehicle group.

Table 1

Effect of nicotinic receptor antagonists on the potency and efficacy of the relaxant effect of carbachol.

	Clasp Fibers		Sling Fibers	
	EC ₅₀ relax (μM)	E _{max} relax (% max Tension)	EC ₅₀ relax (μM)	E _{max} relax (% max Tension)
Vehicle	241 ± 16	66 ± 4.3	310 ± 24 ^{##}	21.5 ± 4.1 [#]
3E-4 decamethonium	382 ± 71	23 ± 12 ^{**}	494 ± 54 [*]	4.6 ± 2.8 [*]
1E-5 mecamylamine	384 ± 84	28 ± 7.1 ^{**}	293 ± 27	8.5 ± 1.5 [*]
1E-4 hexamethonium	292 ± 32	49 ± 4.5	260 ± 28	8.9 ± 2.2 [*]

N and n values for each condition are as shown in figure 4.

^{*}, ^{**} denotes significant difference between antagonist treated and vehicle treated groups,

[#], ^{##} denotes significant difference between clasp and sling fibers (p<0.05 and p<0.01 respectively).