

Proof of your article (# GI-00129-2009) from "American Journal of Physiology-Gastrointestinal and Liver Physiology" is available for download

Dear Sir or Madam:

Please refer to this URL address

<http://rapidproof.cadmus.com/RapidProof/retrieval/index.jsp>

Login: your e-mail address

Password: 77PnpAxT9o77

The file at the above URL address contains the following:

' Proofreading marks

' Reprint Order form

' Copyedited page proof of your article

The site contains 1 file. You will need Adobe Acrobat® Reader to read this file. Adobe Acrobat® Reader is a free software, available for user downloading at <http://www.adobe.com/products/acrobat/readstep.html>.

After you print the PDF file of your paper, please read the page proof carefully and

1) clearly indicate all changes or corrections on the margin;

2) answer all queries (footnotes 1, 2, 3, etc.) listed on the last page of the PDF proof;

3) carefully proofread all/any tables and equations;

4) make sure that any special characters, such as Greek letters, especially μ (mu), have translated correctly;

5) If you have questions about figure quality, note your concerns on the margin of the relevant page. Please keep in mind that the final printed version will be of higher quality than the PDF proof and that the online version of the published article will appear identical.

We encourage you to retain a copy of the proof with your corrections, should further changes and/or clarifications be required.

IMPORTANT NOTES

1. To guarantee the placement of your article in the next available issue of the "American Journal of Physiology-Gastrointestinal and Liver Physiology", please return the corrected set of PDF page proof via an overnight courier service to this address WITHIN 48 HOURS:

The American Physiological Society

"American Journal of Physiology-Gastrointestinal and Liver Physiology" PROOF

9650 Rockville Pike

Bethesda, MD 20814-3991

USA

phone: 301-634-7070

2. Please fax your order form and purchase order to 877-705-1373. Prepayment of checks should be mailed to:

Cadmus Reprints

P.O. Box 822942

Philadelphia, PA 19182-2942

Note: Do not send express packages to this location.

FEIN #:541274108

For reprint inquiries, please contact Pete Brown, fax: 877-705-1373, e-mail: brownp@cadmus.com.

If you have any problems or questions, please contact me.

PLEASE ALWAYS INCLUDE YOUR ARTICLE NO. (GI-00129-2009) WITH ALL
CORRESPONDENCE.

Sincerely,

Ellyn Kestnbaum
Journal Editorial Supervisor
AJP-Gastrointestinal and Liver Physiology

9650 Rockville Pike
Bethesda, Maryland 20814-3991 (USA)
Phone: (301) 634-7185
Fax: 301-634-7244
E-mail: ekestnbaum@the-aps.org

Proofreader's Marks

MARK	EXPLANATION	EXAMPLE
	TAKE OUT CHARACTER INDICATED	Your proof.
^	LEFT OUT, INSERT	u Yor proof. ^
#	INSERT SPACE	# Yourproof. ^
9	TURN INVERTED LETTER	Your p ^l oof. ^
X	BROKEN LETTER	X Your pr/of.
eg#	EVEN SPACE	eg# A good proof.
C	CLOSE UP: NO SPACE	Your pro ^g f.
tr	TRANSPOSE	tr A proof good
wf	WRONG FONT	wf Your proof.
lc	LOWER CASE	lc Your /proof.
≡ caps	CAPITALS	Your proof. caps Your proof.
ital	ITALIC	Your proof. ital Your proof.
rom	ROMAN, NON ITALIC	rom Your proof.
bf	BOLD FACE	Your proof. bf Your proof.
..... stet	LET IT STAND	Your proof. stet Your proof.
out sc.	DELETE, SEE COPY	out sc. She Our proof. ^
spell out	SPELL OUT	spell out Queen (Eliz.)
#	START PARAGRAPH	# read. [Your
no #	NO PARAGRAPH: RUN IN	no # marked. → # Your proof.
L	LOWER	L [Your proof.]

MARK	EXPLANATION	EXAMPLE
┌	RAISE	┌ Your proof.
└	MOVE LEFT	└ Your proof.
┐	MOVE RIGHT	┐ Your proof.
	ALIGN TYPE	└ Three dogs. Two horses.
≡	STRAIGHTEN LINE	≡ Your proof.
⊙	INSERT PERIOD	⊙ Your proof. ^
;/	INSERT COMMA	;/ Your proof. ^
:/	INSERT COLON	:/ Your proof. ^
;/	INSERT SEMICOLON	;/ Your proof. ^
∨	INSERT APOSTROPHE	∨ Your mans proof. ^
∨ ∨	INSERT QUOTATION MARKS	∨ ∨ Marked it proof. ^ ^
=/	INSERT HYPHEN	=/ A proofmark. ^
!	INSERT EXCLAMATION MARK	! Prove it. ^
?	INSERT QUESTION MARK	? Is it right. ^
⊙	QUERY FOR AUTHOR	⊙ was Your proof read by ^
[/]	INSERT BRACKETS	[/] The Smith girl ^ ^
</>	INSERT PARENTHESES	</> Your proof. ^ ^
1/m	INSERT 1-EM DASH	1/m Your proof. ^
□	INDENT 1 EM	□ Your proof
▢	INDENT 2 EMS	▢ Your proof.
▣	INDENT 3 EMS	▣ Your proof.

Gastrointestinal and Liver Physiology 2010

Published by The American Physiological Society

This is your reprint order form or pro forma invoice

(Please keep a copy of this document for your records. This form is not for commercial ordering.)

IMPORTANT Order form must be returned within 48 hours of receipt to avoid late charges. Orders received after 48 hours will be charged an additional fee of 25%. Orders received after 30 days will be charged an additional 50%. It is the policy of Cadmus Reprints to issue only one invoice per order. **Please print clearly. Please return form whether reprints are ordered or not.**

Author Name _____
Title of Article _____
Issue of Journal _____ Reprint # 3577916 Manuscript # GI-00129-2009 Publication Date _____
Number of Pages _____ Color in Article? Yes / No (Please Circle) Symbol AGI
Please include the journal name, the reprint number, and the manuscript number on your purchase order or other correspondence.

Order and Shipping Information

Reprint Costs (Please see page 2 of 2 for reprint costs/fees.)

_____ Number of reprints ordered \$ _____
_____ Number of color reprints ordered \$ _____
Subtotal \$ _____
Add appropriate sales tax/GST to subtotal \$ _____
First address included, add \$32 for each additional shipping address \$ _____

Publication Fees (Please see page 2 for fees and descriptions.)

Page Charges: \$70 per journal page \$ _____
Color Figures: \$400 per color figure \$ _____
Hard copy color proof: \$75 per figure \$ _____
Toll-Free Link: \$150 \$ _____

Member No. _____ Member Signature _____
Total Publication Fees \$ _____
TOTAL TO REMIT \$ _____

Shipping Address (cannot ship to a P.O. Box)

Name _____
Institution _____
Street _____
City _____ State _____ Zip _____
Country _____
Quantity _____ Fax _____
Phone: Day _____ Evening _____
E-mail Address _____

Additional Shipping Address* (cannot ship to a P.O. Box)

Name _____
Institution _____
Street _____
City _____ State _____ Zip _____
Country _____
Quantity _____ Fax _____
Phone: Day _____ Evening _____
E-mail Address _____

* Add \$32 for each additional shipping address

Payment and Credit Card Details (FEIN #:540157890)

Enclosed: Personal Check _____
Institutional Purchase Order _____
Credit Card Payment Details _____
Checks must be paid in U.S. dollars and drawn on a U.S. Bank.
Credit Card: ___ VISA ___ Am. Exp. ___ MasterCard
Card Number _____
Expiration Date _____
Signature: _____
Name (please print): _____

Wire Transfer Payment Information:

PNC Bank
Two Tower Center Boulevard
East Brunswick, NJ 08816
Account Name: Cadmus, a Cenveo Company
ABA/Routing #: 031207607
Account #: 8026256369 ; SWIFT Code: PNCCUS33
Reference #: 822942/Invoice Number OR Reprint/Man #

Invoice or Credit Card Information

Please complete as it appears on credit card statement. Cadmus will process credit cards and *Cadmus Journal Services* will appear on the credit card statement. **Please Print Clearly**

Name _____
Institution _____
Department _____
Street _____
City _____ State _____ Zip _____
Country _____
Phone _____ Fax _____
E-mail Address _____

Please **fax** your order form and purchase order to 877-705-1373. Or, in lieu of faxing, you may **email** the order form and purchase order directly to BrownP@cadmus.com. **Checks** should be mailed to address below:

Cadmus Reprints
P.O. Box 822942
Philadelphia, PA 19182-2942

FEIN #:540157890

Note: Do not send express packages to this location, PO Box.

SIGNATURE REQUIRED: By signing this form the author agrees to accept responsibility for the payment of the mandatory page charges of \$70 per page, reprints ordered, as well as any color charges, late payments, and split shipment charges. If the charges are billed to an institution, the author must assume the responsibility for making the necessary arrangements for the issuance of a formal institutional purchase order. Otherwise, it is understood that the author will bear the cost of these charges. Failure to pay any of these agreed-upon charges could jeopardize future submissions.

AUTHOR Signature _____ Fax _____
Telephone _____ E-mail _____

Gastrointestinal and Liver Physiology 2010

Published by The American Physiological Society

REPRINT AND PUBLICATION CHARGES; Author rates only. Not to be used for commercial ordering

Black and White Reprint Prices

Black and White Pricing, Domestic (USA Only)					
# of Pages	100	200	300	400	500
1-4	\$250	\$349	\$446	\$546	\$643
5-8	\$339	\$511	\$688	\$862	\$1,034
9-12	\$436	\$664	\$897	\$1,122	\$1,351
13-16	\$523	\$827	\$1,132	\$1,436	\$1,743
17-20	\$609	\$982	\$1,351	\$1,724	\$2,091
21-24	\$708	\$1,145	\$1,578	\$2,015	\$2,450
25-28	\$794	\$1,308	\$1,820	\$2,331	\$2,842
29-32	\$897	\$1,471	\$2,061	\$2,648	\$3,236

Black and White Pricing, International (non-USA Only)					
# of Pages	100	200	300	400	500
1-4	\$280	\$393	\$509	\$623	\$738
5-8	\$384	\$588	\$795	\$1,001	\$1,205
9-12	\$499	\$771	\$1,055	\$1,324	\$1,604
13-16	\$601	\$967	\$1,335	\$1,703	\$2,073
17-20	\$702	\$1,154	\$1,604	\$2,053	\$2,497
21-24	\$815	\$1,345	\$1,876	\$2,407	\$2,937
25-28	\$920	\$1,541	\$2,162	\$2,787	\$3,405
29-32	\$1,035	\$1,738	\$2,454	\$3,168	\$3,882

Minimum order is 100 copies. For orders larger than 500 copies, please consult Cadmus Reprints at 410-943-0629.

Late Order Charges

Articles more than 90 days from publication date will carry an additional charge of \$5.98 per page for file retrieval.

Page Charges

\$70 per journal page for all pages in the article, whether or not you buy reprints.

Color

Reprints containing color figures are available. If your article contains **color**, you must pay subsidized color charges of \$400/fig. (reprint charge is \$1000/fig for those who do not pay promptly), whether or not you buy reprints. These **color charges are waived for APS Members who are the first or last author of the paper**. If you requested a **hard copy color figure proof** when you reviewed your S-proof, the charge is \$75.

Shipping

Shipping costs are included in the reprint prices. Domestic orders are shipped via FedEx Ground service. Foreign orders are shipped via an expedited air service. The shipping address printed on an institutional purchase order always supersedes.

Multiple Shipments

Orders can be shipped to more than one location. Please be aware that it will cost \$32 for each additional location.

State Sales Tax and Canadian GST

Residents of Virginia, Maryland, Pennsylvania, and the District of Columbia are required to add the appropriate sales tax to each reprint order. For orders shipped to Canada, please add 5% Canadian GST unless exemption is claimed.

Color Reprint Prices

Color Pricing, Domestic (USA Only)					
# of Pages	100	200	300	400	500
1-4	\$365	\$581	\$794	\$1,009	\$1,222
5-8	\$455	\$743	\$1,035	\$1,325	\$1,613
9-12	\$552	\$896	\$1,243	\$1,584	\$1,931
13-16	\$639	\$1,059	\$1,481	\$1,899	\$2,322
17-20	\$725	\$1,214	\$1,618	\$2,187	\$2,668
21-24	\$824	\$1,376	\$1,834	\$2,478	\$3,028
25-28	\$909	\$1,539	\$2,064	\$2,794	\$3,422
29-32	\$1,011	\$1,702	\$2,294	\$3,110	\$3,815

Color Pricing, International (non-USA Only)					
# of Pages	100	200	300	400	500
1-4	\$397	\$625	\$858	\$1,087	\$1,319
5-8	\$501	\$821	\$1,145	\$1,466	\$1,787
9-12	\$615	\$1,004	\$1,405	\$1,790	\$2,187
13-16	\$717	\$1,201	\$1,685	\$2,170	\$2,657
17-20	\$820	\$1,388	\$1,955	\$2,521	\$3,082
21-24	\$932	\$1,580	\$2,228	\$2,876	\$3,523
25-28	\$1,038	\$1,777	\$2,515	\$3,257	\$3,991
29-32	\$1,154	\$1,974	\$2,808	\$3,638	\$4,471

TOLL-FREE LINK

A link can be created from a url of your choice to your article online so that readers accessing your article from your url can do so without a subscription. The cost is \$150. This is especially useful if your article contains electronic supplemental material. For more information, please click on this link:

<http://www.the-aps.org/publications/sprooflink.pdf>

Ordering

Please **fax** your order form and purchase order to 877-705-1373. Or, in lieu of faxing, you may **email** the order form and purchase order directly to BrownP@cadmus.com. **Checks** should be mailed to address below:

Cadmus Reprints
P.O. Box 822942
Philadelphia, PA 19182-2942

FEIN #:540157890

Note: Do not send express packages to this location, PO Box.

Wire Transfer Payment Information:

PNC Bank
Two Tower Center Boulevard
East Brunswick, NJ 08816
Account Name: Cadmus, a Cenvo Company
ABA/Routing #: 031207607
Account #: 8026256369 ; SWIFT Code: PNCCUS33
Reference #: 822942/Invoice Number OR Reprint/Man #

Please direct all inquiries to:

Pete Brown
866-487-5625 (toll free)
410-943-3095 (direct)
877-705-1373 (FAX)
brownp@cadmus.com

Reprint Order Forms and Purchase Orders or prepayments must be received 48 hours after receipt of form.

Please return this form even if no reprints are ordered.

Comparison of human and porcine gastric clasp and sling fiber contraction by M₂ and M₃ muscarinic receptors

AQ: 1

Anil K. Vegesna,¹ Alan S. Braverman,² Larry S. Miller,¹ Ronald J. Tallarida,^{3,4} Mansoor I. Tiwana,¹ Umar Khayyam,¹ and Michael R. Ruggieri, Sr.^{2,3}

Departments of ¹Medicine, ²Urology, and ³Pharmacology and ⁴Center for Substance Abuse, Temple University School of Medicine, Philadelphia, Pennsylvania

Submitted 2 April 2009; accepted in final form 2 February 2010

Vegesna AK, Braverman AS, Miller LS, Tallarida RJ, Tiwana MI, Khayyam U, Ruggieri MR. Comparison of human and porcine gastric clasp and sling fiber contraction by M₂ and M₃ muscarinic receptors. *Am J Physiol Gastrointest Liver Physiol* 298: G000–G000, 2010. First published February 4, 2010; doi:10.1152/ajpgi.00129.2009.—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 with pig sling fibers but lower in human compared with 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 that M₂ and M₃ receptors mediate contraction in pig clasp fibers. Taken together, the data suggest that both M₂ and M₃ muscarinic receptors mediate the contraction in both pig clasp and sling fibers similar to human clasp and sling fibers.

gastroesophageal reflux; receptor immunoprecipitation; smooth muscle contractility; animal models

AQ: 3 GASTROESOPHAGEAL REFLUX DISEASE (GERD) is a common disease with an incidence of ~5 per every 1,000 person-years, and its prevalence is estimated to be 10–15% in the adult Western population (8). GERD is a major health care burden with regard 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 pathophysiological events occurring in GERD appear at first to be simple because the underlying pathogenesis is the movement of gastric contents into the esophageal lumen. However, there is an intense debate surrounding the pathogenesis of GERD with respect to how the protective mechanisms at the gastroesophageal 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 HPZ was recognized (12). A detailed description of the anatomical arrangement of the smooth muscle fibers around the GEJ was published in 1979 (17). This study reported that the muscle fibers at the lesser curvature of the stomach were clasp muscle fibers and that those at the greater curvature were 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 physiological, pathological, and pharmacological attributes of these structures. High-resolution endoscopic ultrasound, esophageal manometry, autopsy, and animal experiments have been utilized over the past three decades 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 vs. sling stomach fibers (22). The clasp and sling fibers were also shown to relax to electric field stimulation, whereas 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 are similar to those of humans (1, 6). In vivo studies in pigs have shown a swallow-induced LE 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 pharmacological 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 LE circular (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 patients with GERD, we found that the proximal pressure profile attributable 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

Address for reprint requests and other correspondence: M. R. Ruggieri, Sr., Temple Univ. School of Medicine, 3400 N. Broad St., 715 OMS, Philadelphia, PA 19140 (e-mail: rugg@temple.edu)

AQ: 7

AQ: 8 Table 1. Comparison of M₂ and M₃ muscarinic receptor density in pig and human clasp and sling fibers

	Pig			Human		
	Total	M ₂	M ₃	Total	M ₂	M ₃
Clasp	797 ± 14	512 ± 17	20 ± 1.3	228 ± 20	116 ± 16	8 ± 2
Sling	856 ± 27*	443 ± 39	32 ± 1.5*	353 ± 7*	171 ± 6	60 ± 14*

Values are means ± SE. Receptor density is expressed as fmol receptors per mg solubilized protein. Values for human tissues were extracted from our previously published results (4). *Significant difference (*P* < 0.05, Student's *t*-test) between clasp and sling fibers.

includes any anatomical or physiological 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 M₂ and individual M₃ muscarinic receptor subtypes in these tissues. Results are compared with our previously reported findings in human clasp and sling fibers.

MATERIALS AND METHODS

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

Animal specimens. Pig tissues were obtained from local slaughter houses. The pigs were 6–12 mo of age and weighed ~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 postslaughter. 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 were as follows (in mM): 125 NaCl, 2.7 KCl, 0.4 NaH₂PO₄, 1.8 CaCl₂, 0.5 MgCl₂, 23.8 NaHCO₃, and 5.6 glucose. The preserved specimens were transported to the laboratory within 1–2 h of collection.

Dissection. 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 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 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 that the orientation of the muscle fibers was 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% O₂-5% CO₂ at 37°C.

Carbachol concentration response curves. Following equilibration to the bath solution for at least 30 min, the strips were challenged with

30 μM carbachol and rinsed with buffer (5 exchanges over 60 min). 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 min in the presence or absence of one of three 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 on the basis of 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 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, Northampton, MA), and Schild plots 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 pK_b ± SE is reported. If the 95% confidence interval of the slope of the Schild plot did not include 1, pA₂ ± SE 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 10 μg/ml of the following protease inhibitors: soybean and lima bean trypsin inhibitors, aprotinin, leupeptin, pepstatin, and α2-macroglobulin. A sample (20 μl) of [³H]quinuclidinyl benzilate (QNB) (49 Ci/mM, ~4,000 cpm/μl) per milliliter 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 protease inhibitors at 100 mg wet weight per milliliter. Samples were incubated for 50 min at 4°C, with inversion every 5 min 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 M₂ antibody, the M₃ 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 200 μl 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. A sample (50 μl) of 72.5 mM deoxycholate/750

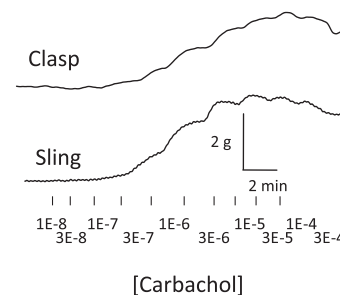


Fig. 1. Original tracings of carbachol concentration response experiments from pig clasp and sling muscle fibers. AQ: 6

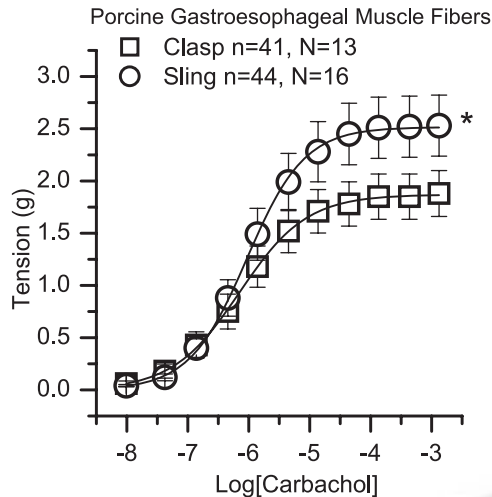


Fig. 2. Carbachol concentration response curves for porcine clasp and sling fibers. *Denotes statistically significant difference in maximal contraction between clasp and sling fibers ($P < 0.05$, Mann-Whitney U -test), n = number of muscle strips, N = number of pigs.

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 μ l of 1 M HCl. Radioactive counts were determined by liquid scintillation spectrometry. Protein content was determined by a Co-

massie blue dye binding protein assay using bovine serum albumin as a standard. Receptor density (means \pm SE) is reported as femtomoles (fmol) receptor per milligram of solubilized protein.

RESULTS

Immunoprecipitation. The results of the receptor density determinations are shown in Table 1 compared with our previous results in human tissue (4). As in the human tissue, the total and M₃ receptor density in the pig sling fibers were 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 shown in Fig. 1. As seen in Fig. 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 (Fig. 3, A and C) and sling

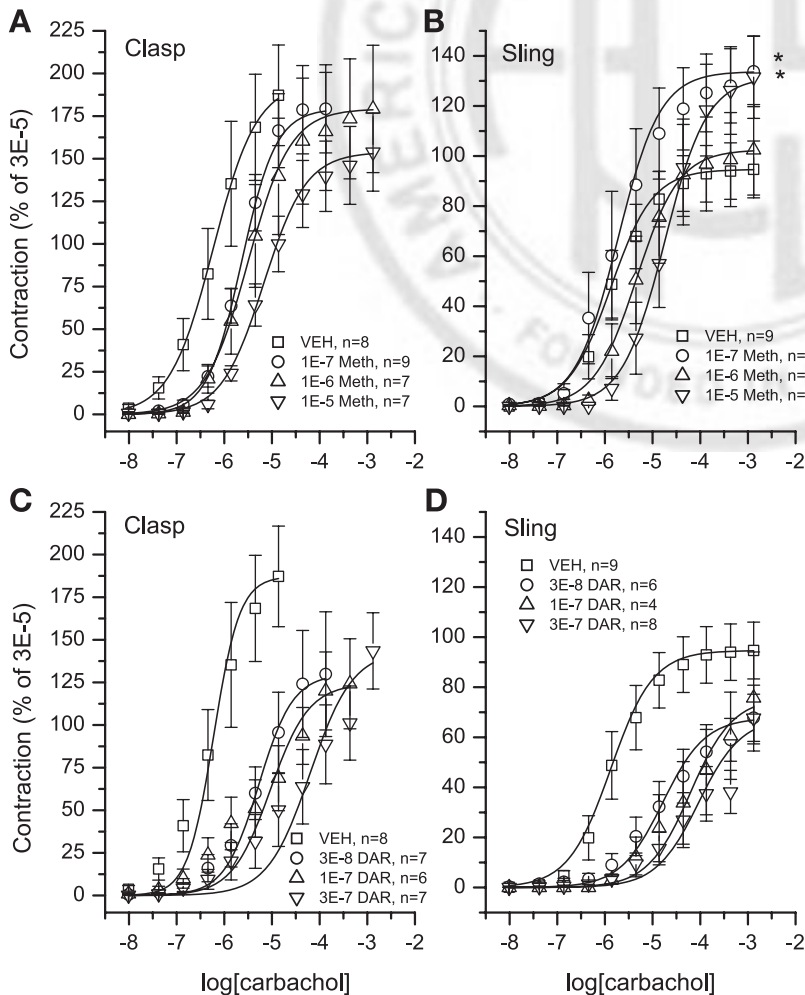


Fig. 3. Concentration response curves for carbachol-induced contraction of clasp (A and C) and sling fibers (B and D) in the presence of various concentrations of methoctramine (Meth) (A and B) and darifenacin (DAR) (C and D). Results are shown as percent of the initial contractile response induced by 30 μ M 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).

fibers (Fig. 3, B and D). Also shown in Fig. 3 are the curves for graded doses of this agonist with three different fixed concentrations of darifenacin, a relatively selective M₃ competitive antagonist, and three different fixed concentrations of methoctramine, an M₂ selective antagonist. Schild plots for determining the potency of the antagonists to inhibit contraction are shown in Fig. 4. In clasp fibers, the intermediate potency of darifenacin (pK_b = 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 (pK_b = 8.6) suggests 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 present 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 pharmacological analysis of concentration-effect relationships was formulated before the concept of multiple receptor subtypes existed and is based on 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. On the basis of the result that darifenacin is less potent in inhibiting clasp fiber 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 E_{max} 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 inhibit the signaling from M₃ receptors in the muscle. This would result in a subadditive interaction. An increased contractile response could occur as a consequence of blocking the M₂ receptor inhibition on M₃ signaling by methoctramine.

F4

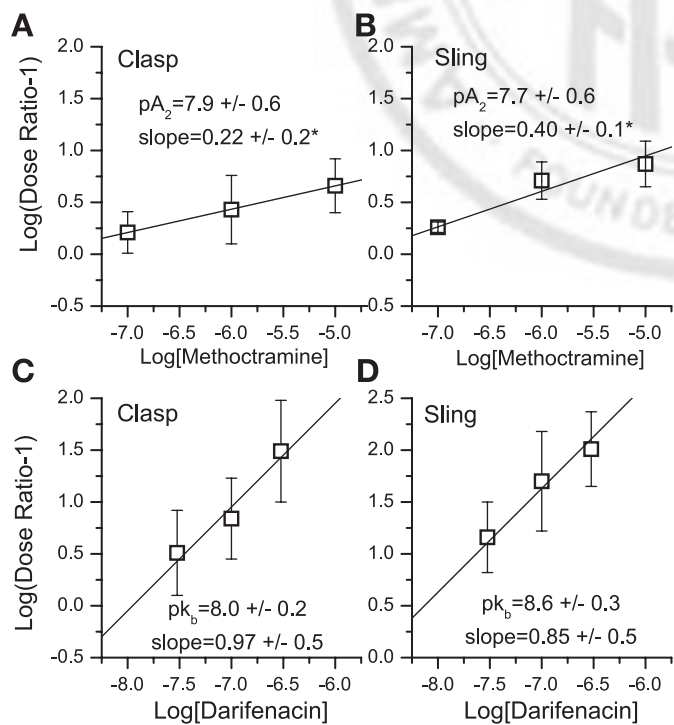


Fig. 4. Schild plots for determination of the potency of methoctramine (A and B) and darifenacin (C and D) for inhibition of carbachol-induced contraction of porcine clasp (A and C) and sling (B and D) fibers. *Denotes that the slope of the Schild plot is significantly less than unity.

Because of 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 present 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 need to 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 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.

DISCLOSURES

AQ: 4 No conflicts of interest are declared by the authors.

REFERENCES

1. Aggestrup S, Uddman R, Jensen SL, Hakanson R, Sundler F, Schafalitzky de Muckadell O, Emson P. Regulatory peptides in lower esophageal sphincter of pig and man. *Dig Dis Sci* 31: 1370–1375, 1986.
2. Brasseur JG, Ulerich R, Dai Q, Patel DK, Soliman AMS, Miller LS. Pharmacological dissection of the human gastro-oesophageal segment into three sphincteric components. *J Physiol* 580: 961–975, 2007.
3. Braverman AS, Lebed B, Linder M, Ruggieri MR. M₂ mediated contractions of human bladder from organ donors is associated with an increase in urothelial muscarinic receptors. *NeuroUrol Urodyn* 26: 63–70, 2007.
4. Braverman AS, Miller LS, Vegesna AK, Tiwana MI, Tallarida RJ, Ruggieri MR Sr. Quantitation of the contractile response mediated by two receptors: M₂ and M₃ muscarinic receptor mediated contractions of human gastroesophageal smooth muscle. *J Pharmacol Exp Ther* 329: 218–224, 2009.
5. Braverman AS, Tallarida RJ, Ruggieri MR Sr. The use of occupation isoboles for analysis of a response mediated by two receptors: M₂ and M₃ muscarinic receptor subtype-induced mouse stomach contractions. *J Pharmacol Exp Ther* 325: 954–960, 2008.
6. Brown DR, Timmermans JP. Lessons from the porcine enteric nervous system. *Neurogastroenterol Motil* 16, Suppl 1: 50–54, 2004.
7. Burleigh DE. The effects of drugs and electrical field stimulation on the human lower oesophageal sphincter. *Arch Int Pharmacodyn Ther* 240: 169–176, 1979.
8. Camilleri M, Dubois D, Coulie B, Jones M, Kahrilas PJ, Rentz AM, Sonnenberg A, Stanghellini V, Stewart WF, Tack J, Talley NJ, Whitehead W, Revicki DA, Camilleri M, Dubois D, Coulie B, Jones M, Kahrilas PJ, Rentz AM, Sonnenberg A, Stanghellini V, Stewart WF, Tack J, Talley NJ, Whitehead W, Revicki DA. Prevalence and socioeconomic impact of upper gastrointestinal disorders in the United States: results of the US Upper Gastrointestinal Study. *Clin Gastroenterol Hepatol* 3: 543–552, 2005.
9. Carsi JM, Valentine HH, Potter LT. m₂-Toxin: a selective ligand for M₂ muscarinic receptors. *Mol Pharmacol* 56: 933–937, 1999.
10. Caulfield MP. Muscarinic receptors—characterization, coupling and function. *Pharmacol Ther* 58: 319–379, 1993.
11. Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* 50: 279–290, 1998.
12. Code CF, Fyke FE Jr, Schlegel JF. The gastroesophageal sphincter in healthy human beings. *Gastroenterologia* 86: 135–150, 1956.
13. Evans T, Hepler JR, Masters SB, Brown JH, Harden TK. Guanine nucleotide regulation of agonist binding to muscarinic cholinergic receptors. Relation to efficacy of agonists for stimulation of phosphoinositide breakdown and Ca²⁺ mobilization. *Biochem J* 232: 751–757, 1985.
14. Gaddum J. The Quantitative effects of antagonistic drugs. *J Physiol* 89: 7P–9P, 1937.
15. Korn O, Stein HJ, Richter TH, Liebermann-Meffert D. Gastroesophageal sphincter: a model. *Dis Esophagus* 10: 105–109, 1997.
16. Lerche W. *The Esophagus and Pharynx in Action. A Study of Structure in Relation to Function*. Springfield, IL: Charles C. Thomas, 1950.
17. Liebermann-Meffert D, Allgower M, Schmid P, Blum AL. Muscular equivalent of the lower esophageal sphincter. *Gastroenterology* 76: 31–38, 1979.
18. McCray WH Jr, Chung C, Parkman HP, Miller LS. Use of simultaneous high-resolution endoluminal sonography (HRES) and manometry to characterize high pressure zone of distal esophagus. *Dig Dis Sci* 45: 1660–1666, 2000.
19. McKinney M, Miller JH, Gibson VA, Nickelson L, Aksoy S. Interactions of agonists with M₂ and M₄ muscarinic receptor subtypes mediating cyclic AMP inhibition. *Mol Pharmacol* 40: 1014–1022, 1991.
20. Miller LS, Dai Q, Vegesna A, Korimilli A, Ulerich R, Schiffner B, Brasseur JG. A missing sphincteric component of the gastro-esophageal junction in patients with GERD. *Neurogastroenterol Motil*. In Press.
21. Schopf BW, Blair G, Dong S, Troger K. A porcine model of gastroesophageal reflux. *J Invest Surg* 10: 105–114, 1997.
22. Tian ZQ, Liu JF, Wang GY, Li BQ, Wang FS, Wang QZ, Cao FM, Zhang YF. Responses of human clasp and sling fibers to neuromimetics. *J Gastroenterol Hepatol* 19: 440–447, 2004.
23. Vicente Y, Da Rocha C, Yu J, Hernandez-Peredo G, Martinez L, Perez-Mies B, Tovar JA. Architecture and function of the gastroesophageal barrier in the piglet. *Dig Dis Sci* 46: 1899–1908, 2001.

AQ: 5

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES

1

AQ1— Please read these proofs carefully, print them out, answer any queries, make any changes in the margin, mail them back in the next 48 hours to AJP Gastrointestinal and Liver Physiology PROOF, 9650 Rockville Pike, Bethesda, MD 20814. Please make a copy of proof for your records. We are close to proofreading deadline, so please do not delay. Thanks.

AQ7— Please verify accuracy of your e-mail address in the address for correspondence, or delete e-mail address if you do not want it included (Note: this material is listed as a footnote at bottom of left column of text, on the first page.)

AQ2— Please note that the author list in the abstract line represents the form in which these names will appear in many online databases, such as the NCBI/NIH/NLM Pubmed database. Check this carefully and be sure there are no misrepresentations. Please make a note on the proof if any corrections are needed.

AQ3— “~5 per every 1,000 person-years” stated as meant?

AQ8— Ed: Table and figure have been moved ahead of cite to avoid reference page.

AQ6— If applicable, please define E in Figure 1.

AQ4— Please verify that the DISCLOSURES statement is accurate and truthful.

AQ5— Please provide the issue info for Ref. 20 if it has become available.
