Functional significance of muscarinic receptor expression within the proximal and distal rat vagina.

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ABSTRACT

Information regarding the role of cholinergic nerves in mediating vaginal smooth muscle contraction is sparse and in vitro studies of the effects of muscarinic agonists on vaginal smooth muscle are discrepant. The goal of this study was to determine the expression of muscarinic receptors in the vaginal wall of the rat. In addition, we sought to determine the effect of the muscarinic receptor agonist, carbachol, on contractility and inositol phosphate production of the proximal and distal rat vaginal muscularis. RT-PCR analysis indicated that both M2 and M3 receptor transcripts were expressed within the proximal and distal rat vagina. Carbachol dose-dependently (10⁻⁷-10⁻⁴ M) contracted the rat vaginal muscularis with a greater maximal contractile response in the proximal vagina (P < 0.01) as compared to the distal vagina. The contractile responses of the rat vaginal muscularis to carbachol were dose-dependently inhibited by the M3 antagonist, p-F-FHSiD and a pK₈ of 7.78 and 7.95 was calculated for the proximal and distal vagina respectively. Inositol phosphate production was significantly increased in both regions of the vagina following 20-min exposure to 50 μM carbachol with higher levels detected in the proximal vagina as compared to the distal (P < 0.05). Preliminary experiments indicated the presence of M2 and M3 receptors in the human vaginal muscularis as well as contraction of human vaginal muscularis to carbachol indicating that our animal studies are relevant to human tissue. Our results provide strong evidence for the functional significance of M3 receptor expression in the vaginal muscularis.
Keywords: vagina, smooth muscle, muscarinic receptor, female sexual response
INTRODUCTION

The vagina receives parasympathetic and sympathetic innervation via the pelvic nerves, hypogastric nerves, and sympathetic chain ganglia (17). Although it has been well documented that sympathetic nerves regulate vaginal smooth muscle function, the role of parasympathetic nerves in mediating vaginal smooth muscle contraction has received limited attention. In light of the high prevalence of pelvic organ prolapse (34) and female sexual dysfunction (24) it is essential to develop a more complete understanding of autonomic innervation of the vagina.

*In vivo* studies of vaginal smooth muscle contraction utilizing pelvic nerve stimulation (PNS) as an animal model of female sexual arousal have provided direct (16) and indirect (27) evidence that the proximal vagina contracts in response to PNS. The distal vagina, however, has been shown to relax in response to PNS (27; 31) suggesting a regional difference in vaginal smooth muscle function. The majority of *in vitro* studies of vaginal smooth muscle have investigated signaling pathways responsible for inducing relaxation of the distal vagina (3; 14; 15; 19; 20; 40). In support of the suggestion that regional differences are present in vaginal smooth muscle responses to PNS, we have provided molecular and functional evidence that the rat proximal vagina is composed of a phasic-type smooth muscle compared to a tonic-type smooth muscle in the distal vagina (5). Together, these studies emphasize the importance of accounting for regional differences in structure and function when studying the vaginal muscularis.
A major pathway of parasympathetic activation of smooth muscle contraction is via interaction of acetylcholine released from cholinergic nerves with muscarinic receptors on muscle cells. Five subtypes of muscarinic receptors (M₁, M₂, M₃, M₄, M₅) have been pharmacologically described and molecularly identified (8). Although M₂ receptors outnumber M₃ receptors within smooth muscle, pharmacological studies have indicated that M₃ receptor signaling is responsible for the majority of muscarinic agonist induced smooth muscle contraction of the lower urogenital tract including the bladder (2) and uterus (1; 10; 23). Recent studies with M₃ receptor knockout mice have provided further evidence that the M₃ receptor plays the predominant role in mediating smooth muscle contraction (25). Activation of M₃ receptors coupled to G₉/₁₁ stimulates calcium dependent smooth muscle contraction through the phosphatidylinositol cascade which results in the generation of inositol triphosphate (IP₃). IP₃ stimulates the release of calcium from intracellular stores and increases myosin light chain (MLC₂₀) phosphorylation, the pivotal event responsible for smooth muscle contraction (33).

Positive staining for vesicular acetylcholine transporter (VACHT) has been reported in nerve terminals of the rat vaginal muscularis (14; 35). Cadaveric studies have also demonstrated an abundance of VACHT-positive terminals within the mid and proximal human vagina (39). These studies suggest cholinergic nerves may be important in mediating vaginal smooth muscle contraction. However, results from in vitro studies of vaginal contraction in response to the muscarinic agonist carbachol have been conflicting (14; 29; 30;
Intracellular signaling pathways linked to M₃ receptor activation have yet to be explored within the vaginal muscularis. The objectives of this study were to clarify the significance of parasympathetic innervation of the vaginal wall by determining the effect of muscarinic receptor stimulation on contractile activity and intracellular signaling events linked to M₃ receptor stimulation of vaginal smooth muscle.
MATERIALS AND METHODS

Animal Studies

Animals: Animal use and the experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Virgin, female Sprague-Dawley rats (250-300 grams; 3-4 months) were obtained from Charles River Laboratory and housed in a temperature (25°C) and light-controlled (12:12-h light-dark) room with free access to food and water. The estrus cycle of rats was monitored by cytological evaluation of vagina smears (28) stained with the Protocol Heme 3 stain set (Fisher Scientific, Pittsburg, PA). Animals were euthanized on the day of estrus by an intraperitoneal injection of an overdose of ketamine (>75mg/kg) and xylazine (>10mg/kg) followed by exsanguination.

Tissue Preparation: The vaginal tube was isolated, dissected from the urogenital tract, cleaned of connective tissue, and cut open longitudinally. The vagina was divided into a proximal (approximately upper two-thirds) and distal segment (approximately lower one-third) under a dissecting microscope as described by Basha et al. (5). The vaginal segments were either snap frozen in liquid nitrogen and stored at -80°C or placed in ice-cold MOPS-buffered physiological salt solution (PSS) for same-day physiological studies. The PSS solution contained (in mM): 140 NaCl, 4.7 KCl, 1.2 MgSO4, 1.6 CaCl2, 1.2 Na2HPO4, 2.0 MOPS, 5.0 D-glucose, and 0.02 Na2-EDTA.

RNA extraction/purification: 50-100 ug of frozen proximal and distal vaginal tissue (n=3) was ground into a fine powder with a liquid nitrogen
prechilled mortar and pestle. RNA was extracted from the powder using TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. RNA concentration was quantified at 260/280 with a UV-visible spectrophotometer (BioSpec-mini, Shimadzu).

*Reverse transcription/Polymerase chain reaction*: Three µg of RNA were reverse transcribed with oligo(dT) primer (Promega, Madison, WI) and Moloney murine leukemia reverse transcriptase (Invitrogen, Carlsbad, CA) at 37° C for 60 minutes. The reaction was stopped by heating to 90° C and the cDNA was stored at -20° C. Polymerase chain reaction (PCR) was performed with 1 µL of cDNA in a 25 µL reaction mixture containing upstream and downstream primer, PCR buffer (Applied Biosystems), dNTP (Roche Applied Science, Germany) and Taq polymerase (Perkin-Elmer, Foster City, CA). Primers for rat M$_2$ and M$_3$ receptors were based on sequences published by Borges et al.(7)

*Isometric Force Measurements:*

**Determination of pEC$_{50}$ and Maximal Response of Proximal and Distal Strips**

Longitudinal strips of proximal and distal rat vagina obtained from 4 animals were mounted between a force transducer (Grass model FT.03) and a stationary clip and immersed in water jacketed organ baths containing PSS (37° C, aerated with 100% O$_2$). Strips were equilibrated for 90 minutes, during which time they were repeatedly contracted by the addition of 110 mM KCl (equimolar substitution for NaCl) to the organ bath. Between contractions, the strips were stretched until the length that approximates the optimal length for maximal active
contraction (L₀) was reached. Carbachol was added to organ baths in a non-cumulative manner to achieve bath concentrations of 10⁻⁷ - 10⁻⁴ M. Each drug addition was washed out of the tissue preparation by repeated washes (3 x 15 minutes) with PSS. Force in response to carbachol was expressed as a % of maximal KCl force development and a sigmoid concentration response curve was constructed using Graph Pad Prism (Ver. 5.02).

**Determination of pKᵦ₈ of p-F- HHSiD**

For studies with the antagonist, p-F-HHSiD, longitudinal strips of proximal and distal rat vagina were mounted, equilibrated and stretched to L₀ as described above. Carbachol was added to organ baths in a cumulative manner to achieve bath concentrations of 10⁻⁷ – 10⁻³ to construct a control dose response curve for each strip. Following a 45 minute wash-out period with PSS, each strip was incubated for 20 minutes in either antagonist or vehicle (time control) and then underwent a second series of cumulative additions of carbachol. Three concentrations of antagonist (30 nM, 100 nM, 300 nM) and time control studies were tested in separate strips obtained from 3-6 animals. A sigmoid dose response curve to carbachol was constructed in the absence (control curve) and presence of antagonist with force normalized to the maximum response of the control curve. The concentration ratio (CR) for each concentration of antagonist was calculated by determining the EC₅₀ in the presence and absence of antagonist for each strip.

**Inositol Phosphate Measurements:** Segments of rat vagina, either proximal or distal (n= 4 animals) , were preincubated for 1 hour in 2 ml PSS
solution at 37°C. The PSS was aerated with 95% O2-5% CO2 until the pH equaled 7.4. After pre-incubation, the segments were incubated for 1 hr in fresh PSS containing [3H] inositol (10 μCi/ml) and then treated for 30 min with LiCl (10 mM). The tissues were then treated with 50 μM carbachol for periods between 2 and 20 min, placed on small pieces of filter paper, and immersed in liquid N2. The frozen segments were thawed in 2 ml cold trichloroacetic acid solution (10% w/v). The trichloroacetic acid solutions were extracted 4 times with 2 ml ethyl ether (4°C) to remove the trichloroacetate. The inositol phosphates were then separated from each other and from free inositol by the procedure of LaBelle and Murray (22). Solutions containing inositol phosphates were applied to columns of Dowex 1X8-400 (200 - 400 mesh) resin (0.5 ml) and washed with 12 ml water to remove the free inositol. The columns were then treated sequentially with 5 ml of 0.2 M, 0.4 M, and 0.8 M ammonium formate/0.1 M formic acid to elute inositol monophosphate (IP1), inositol bisphosphate (IP2) and inositol triphosphate (IP3), respectively. Aliquots (2.5 ml) of each step were added to scintillation fluid (16 ml Ecolume) and the radioactivity in the samples determined using a Beckman scintillation counter.

**Human Studies**

*Human Tissue:* Following approval by the University of Pennsylvania Institutional Review Board, women scheduled to undergo hysterectomy for benign indications (leiomyoma, adenomyosis, or pelvic organ prolapse) were recruited to participate in an ongoing study of human vaginal smooth muscle. Patients with gynecological malignancy, connective tissue disorders, prior
surgery for pelvic organ prolapse, or any neuromuscular disorder were excluded. Following written informed consent, hysterectomy was performed in a standardized fashion and a full thickness vaginal biopsy (posterior) was obtained immediately after removal of the uterus and cervix. Each full thickness biopsy was approximately 1-2 cm (width) x 1 cm (depth) and taken from the central portion of the posterior vaginal cuff as previously described (6).

Tissue preparation: Immediately after surgical excision, biopsies were immersed and stored in Tyrodes buffer (125mM NaCl, 2.7mM KCl, 23.8 mM NaHCO3, 0.5 mM MgCl2.6H2O, 0.4 mM NaH2PO4.H2O, 1.8mM CaCl2, and 5.5 mM dextrose) at 4 ºC to be used later the same day. For the physiology experiments (n=1) and molecular studies (n = 3), the vaginal muscularis was dissected from biopsy samples within 2 hours of excision with the aid of a dissecting microscope. The remainder of the muscularis tissue from the sample used in physiology and all of the muscularis tissue from the other patients (n=3) was snap frozen in liquid nitrogen and stored at -80 ºC for subsequent molecular studies.

RNA extraction/purification: RNA was extracted from frozen pulverized vaginal muscularis samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. To increase RNA yield, 200 g of glycogen was added to each sample and the mixture was passed two times through a 26 gauge needle (to shear genomic DNA) prior to centrifugation at 12,000 g X 10 minutes in 4ºC. RNA concentration was measured at 260/280 wavelengths with a UV-visible spectrophotometer (BiosSpec-mini, Shimadzu).
Reverse Transcription/Polymerase chain reaction:

Two micrograms of RNA underwent RT-PCR as described in the animal studies section. RT-PCR of mRNA transcripts for M₂ and M₃ receptors was performed using primers based on the sequences published by Wang et al. (37).

Isometric Force Measurements: Longitudinal vaginal muscularis strips (~3 mm X 10 mm, ~80-120 mg) were suspended in 15 ml of Tyrodes buffer at 37°C and equilibrated with 95% oxygen -5% CO₂. One end of each muscle strip was attached to a glass rod that was immersed in Tyrodes buffer and the other end was attached to a force transducer. After a 45 minute incubation period, the length of optimal force development (L₀) was determined by increasing the length of each strip in ~ 1.5 mm increments until maximal contractile force to a high KCl solution (125 mM) was achieved. Contractile responses were recorded in response to the cumulative addition of carbachol (1⁻¹⁻ M).

Statistics: All statistics were performed using the Systat Statistical software program (Ver.3.5). pEC₅₀ and maximal response to carbachol were compared using paired t-tests. Generation of inositol phosphates (IP₁, IP₂, IP₃) in response to carbachol were compared using repeated-measures two way – ANOVA. Significant factors (α < 0.05) underwent post-hoc analysis using a Student Neumen-Keul’s test.
RESULTS

RT-PCR analysis indicated that both M2 and M3 receptor transcripts were present in the proximal and distal rat vaginal wall (Figure 1). Non-cumulative addition of carbachol (10^{-7} – 10^{-4} M) contracted the proximal and distal vagina in a concentration dependent manner indicating a functional significance of muscarinic receptor transcript expression in the vaginal wall (Figure 2). The calculated pEC_{50} of the proximal vagina was not significantly different than the distal vagina with an overall mean pEC_{50} of 5.70 ± 0.20. However, the maximal force of contraction of the proximal vagina expressed as a percentage of KCl contraction (172.77 ± 8.83 %) was greater (P < 0.01) than that of the distal vagina (113.62 ± 8.27 %).

In order to examine the significance of M3 receptor expression within the vagina, we evaluated the effects of the selective M3 antagonist, p-F-HHSiD on carbachol induced contractions. Time control experiments indicated that the pEC_{50} and maximal response to cumulative addition of carbachol were not significantly different between two consecutive curves for both the proximal and distal vagina (n= 4). Incubation of strips in p-F-HHSiD (n= 4-7 strips from different animals) dose dependently antagonized carbachol induced contractions and resulted in parallel rightward shifts of the concentration-response curves for both the proximal (Figure 3A) and distal vagina (Figure 3B). Concentration ratios (CR) at each concentration of antagonist were utilized to construct a Schild plot to determine the affinity estimate of p-F-HHSiD (4). The slope of the linear regression of log (CR-1) and log [antagonist] did not deviate significantly from
unity for both proximal (0.99 ± 0.26) and distal (0.82 ± 0.16) strips and therefore the slopes of the lines were constrained to 1. The x-axis intercept of the line estimated a pK₈ of 7.78 (95% C.I. 8.96-7.41) and 7.95 (95% C.I. 8.74-.7.61) for p-F-HHSiD within the proximal and distal vagina respectively (Figure 3C).

In experiments with a separate group of animals (n= 4), incubation of strips in 50 μM carbachol resulted in a significant increase of IP₁, IP₂, and IP₃ (Figure 4A,B,C, respectively) at both 10 and 20 minutes incubation compared to basal levels for proximal strips (P < 0.001) and at 20 minutes incubation for distal strips (P < 0.05). Significantly greater amounts of IP₁, IP₂, and IP₃ were detected in proximal vaginal strips as compared to distal vaginal strips at both 10 and 20 minutes of carbachol stimulation (P < 0.01).

Vaginal muscularis from three patients (shown in Table 3) undergoing hysterectomy were included to demonstrate the presence of the M₂ and M₃ receptor mRNA. Patient demographics are shown in Table 1. All samples were used for molecular and physiologic analyses. Results of RT-PCR indicated that M₂ and M₃ receptor transcripts were expressed within human vaginal muscularis obtained from biopsies taken from the vaginal cuff (Figure 5A). Preliminary functional studies detected a dose dependent contractile response of human vaginal muscularis to the cumulative addition of carbachol with an pEC₅₀ of 3.99 (Figure 5B).
DISCUSSION

Our study is the first report of M₂ and M₃ receptor transcript expression in the rat and human vagina (Figure 1, Figure 5A). In addition, we show that the muscarinic agonist, carbachol, stimulates contraction of both the rat and human vagina with a concomitant increase in inositol phosphate production in the rat vaginal wall. These results suggest that parasympathetic innervation of the vaginal wall plays a significant role in mediating vaginal smooth muscle contraction.

Carbachol (10⁻⁷ – 10⁻⁴ M) contracted both the proximal and distal rat vagina in a concentration dependent manner (Figure 2). Our animal studies indicated that the calculated pEC₅₀ for carbachol induced contraction was not significantly different between the proximal and distal vagina suggesting a similar receptor affinity in both regions of the vagina. Our reported overall mean pEC₅₀ of 5.70 ± 0.20 is similar to values reported for carbachol induced contractions of the rat uterus (10) and female rat bladder (21). In contrast, the maximal contractile response of the proximal vagina was approximately 1.5 times greater than that of the distal vagina. As we expressed the amplitude of carbachol induced contraction as a percentage of KCl contraction, this difference is not due to the greater amount of muscle within the proximal vagina compared to the distal vagina (5) and may suggest either a greater number of muscarinic receptors or a stronger coupling of receptor binding to intracellular events within the proximal vagina. Although this study provides quantitative evidence of M₂ and M₃ receptor transcript expression within the rat and human vagina, quantitative real- time
PCR and binding studies are needed to help determine possible differences in receptor distribution within the proximal and distal vagina.

In general, results of previous studies examining vaginal responses to carbachol have been mixed and contradictory. Several in vitro studies of vaginal contractility have reported a lack of carbachol induced force of both the proximal and distal vagina of the rabbit (29), the mid and proximal human vagina (36) and the proximal and distal rat vagina (30) despite utilizing similar ranges of agonist concentration as that used in the present study. Species differences and differences in strip preparation may explain why others have failed to detect a contractile response of vaginal strips to carbachol. Most relevant for comparison to this study, Onol et al. (30) utilized circular vaginal strips of the rat and did not control for the stage of estrus cycle compared to our study utilizing longitudinal strips taken from rats at the estrus stage of their cycle.

Giraldi et al. (14) on the other hand, demonstrated that carbachol dose dependently contracted the distal vaginal smooth muscle of the rat in agreement with the present study. In contrast to our study, these researchers were unable to detect a contractile response of the proximal vagina to KCl stimulation. This discrepancy is likely due to differences in stretching protocols. Whereas we have estimated $L_0$ by repeated contractions of KCl following successive stretches until developed tension was maximal, Giraldi et al. (14) pre-stretched proximal and distal strips to the same predetermined passive tension of 4-5 mN. It is possible that this stretching protocol may have resulted in a resting length of the proximal vagina that is not within the range of $L_0$. 
We detected carbachol induced contraction of the human vaginal muscularis in response to $10^{-5} - 10^{-3}$ M carbachol (Fig 5B) and mRNA transcripts for M2 and M3 receptors (Fig 5A) suggesting that the results of our animal studies are relevant to the human. We acknowledge that as we were only able to obtain viable tissue from a single biopsy taken from the vaginal cuff of a patient undergoing surgery for utero-vaginal prolapse to date, and therefore this result is preliminary. We did not detect contraction in response to doses less than 10uM and calculated a pEC$_{50}$ of 3.991 which is considerable lower than the mean pEC$_{50}$ for carbachol utilizing rat vaginal strips. This may represent a species difference in sensitivity to carbachol. It is also possible the decreased sensitivity of the human vaginal muscularis to carbachol is due to compromised contractile function of the vaginal muscularis due to prolapse, tissue damage during biopsy collection, and time lapse between biopsy collection and physiological experiments. The influence of biopsy collection procedures and tissue storage may also explain the failure of Uckert et al.(36) to detect a contractile response to carbachol of the human vaginal muscularis. Both our study and the study of Uckert et al. (36) utilized strips taken from menopausal patients. As there is evidence from animal studies that estrogen alters carbachol induced contraction of bladder smooth muscle, it is also possible that menopausal status influences vaginal smooth muscle responses to carbachol. Studies are needed to examine the possible effect of prolonged ovarian hormone loss on muscarinic receptor mediated vaginal smooth muscle contraction.
Our finding that carbachol-induced force was dose dependently inhibited by p-F-HHSiD lends further support that muscarinic receptors regulate vaginal smooth muscle contraction. Our calculated pK₈ values of 7.78 and 7.95 for p-F-HHSiD obtained with strips taken from the proximal and distal vagina, respectively are within the range of potencies (7.5-7.9) previously reported for p-F-HHSiD at the M₃ receptor (See Ref.11 for review). This finding is consistent with studies on nonvascular smooth muscles which indicate that contraction is mediated predominantly via M₃ receptor stimulation (1; 9; 10; 18; 23; 38).

Given the considerable discrepancy in results of in vitro contractile studies of the vaginal wall, and to assist in clarification of the role of muscarinic receptors in mediating contraction of vaginal smooth muscle, we examined the effect of carbachol stimulation on inositol phosphate production of the proximal and distal vagina. It has been widely accepted that M₃ receptors couple to Gq to activate PLC and generate IP₃ leading to calcium dependent smooth muscle contraction. Results of inositol phosphate production in response to 50 µM carbachol indicated that the contractile effects of muscarinic receptor activation may be at least partially attributed to the generation of IP₃. Significant increases in IP₃ and its breakdown products, IP₂ and IP₁, were measured in proximal and distal rat vaginal strips following incubation with carbachol (Figure 4). In addition, our report of significantly greater levels of inositol phosphate production by the proximal vagina as compared to the distal vagina is consistent with our finding of a greater maximal contractile response of the proximal vagina as compared to the distal vagina. As it has been previously reported that PLC activity, and
inositol phosphate production in response to carbachol stimulation are increased during gestation and 17-β estradiol within the rat myometrium, it would be interesting to investigate the possibility of estrogen regulation of intracellular signaling pathways linked to M₃ Receptor activation within the vaginal muscularis.

It should be noted however, that there have been controversial findings indicating that the PLC-IP₃ pathway may not be important in mediating rat urinary bladder contraction based on studies using the PLC inhibitor, U73122 (12; 32). Conversely, Frei et al.(13) have recently demonstrated that U73122 inhibited carbachol induced contractions and calcium signals in the mouse bladder. However, the results suggest that PLC/IP₃ mediated calcium release makes only a minor contribution to muscle contraction, with calcium entry through L-type calcium channels playing a bigger role in M₃ mediated contraction. These results however may be tissue and species specific as McCarron et al. (26) demonstrated that blocking the IP₃ receptor resulted in approximately a 70% reduction in amplitude of carbachol induced contractions of the guinea pig distal colon.

It is important to note that the majority of in vitro and in vivo studies of the vagina have been aimed at identifying mechanisms responsible for distal vaginal relaxation that is reported to occur during the arousal phase of the female sexual response. The results of our current study, in conjunction with our previous report that the proximal vagina is structurally and functionally distinct from the distal vagina (5) suggests that results obtained using the distal vagina can not be
extrapolated to the proximal vagina. Indeed, studies utilizing rodent models of
female sexual arousal have indicated that the proximal vagina contracts in
response to PNS as opposed to relaxation of the distal vagina providing \textit{in vivo}
evidence that the proximal and distal vagina play distinct roles in the female
sexual response. Lastly, the finding that proximal vaginal contractions induced
by PNS were blocked by the muscarinic antagonist atropine (16) is in support of
our evidence of a functional significance of muscarinic receptor expression in the
rat and human vaginal wall.

\textbf{PERSPECTIVES AND SIGNIFICANCE}

Although autonomic regulation of the male sexual response has been well
studied, little is known regarding the neural regulation of the female sexual
response. Our study provides convincing evidence that muscarinic receptor
activation contracts vaginal smooth muscle and suggests that the vaginal
component of the female sexual response may be mediated in part by
parasympathetic nerves. As we have shown regional differences in vaginal
responses to carbachol, we also provide further evidence that the separate
embryological origins of the proximal and distal vagina may result in distinct
functional roles of these two regions of the vagina. Research is needed to more
completely determine the vaginal changes that occur during the female sexual
response and to identify the intracellular signaling cascades responsible for
mediating these changes.
ACKNOWLEDGEMENTS:

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6. **Boreham MK, Wai CY, Miller RT, Schaffer JI and Word RA.**


**Figure 1:** Representative ethidium bromide stained agarose gel of mRNA transcripts amplified by RT-PCR utilizing primers specific for muscarinic receptor subtype 2 (M₂) and muscarinic receptor subtype 3 (M₃). Both M₂ (Panel A) and M₃ (Panel B) receptor transcripts were detected in the proximal and distal rat vaginal wall. Rat brain was utilized as a positive control. R1: rat 1, R2: rat 2.

**Figure 2:** Isometric contraction of longitudinal strips of proximal and distal rat vagina in response to the non-cumulative addition of carbachol (10⁻⁷-10⁻⁴ M). Carbachol stimulated force was expressed as a percentage of maximal 110mM KCl stimulated contraction. The pEC₅₀ was not significantly (N.S.) different between proximal and distal strips, whereas the maximum contractile response of proximal strips was significantly greater (** P < 0.01) than that of the distal strips. Each point represents the mean ± standard deviation (n= 4 animals).

**Figure 3:** The effect of ρ-F-HHSiD, a selective muscarinic receptor subtype 3 antagonist, on the isometric contractile response of longitudinal strips of the proximal and distal vagina to carbachol (10⁻⁷-10⁻³.₅ M). Concentration-response curves for carbachol induced contractions of proximal (A) and distal (B) vaginal strips incubated in vehicle (control curve) and ρ-F-HHSiD (30, 100, 300 nM). C. Schild analysis for ρ-F-HHSiD with proximal and distal strips. A pKB of 7.78 (95 % C.I. 8.96-7.41) and 7.94 (95% C.I. 8.74 – 7.61) was calculated for ρ-F-HHSiD in experiments utilizing proximal and distal strips respectively. Each point represents the mean ± standard deviation (n= 3-6 animals).
Figure 4: Time course of inositol phosphate production of proximal and distal rat vaginal strips in response to 50 uM carbachol. [$^3$H] inositol labeled vaginal strips were treated with 50 uM carbachol for 2, 10 or 20 minutes and individual inositol phosphates were separated by Dowex chromatography. Results are expressed as a percentage of stimulated over control values (time 0). Proximal vaginal strips produced significantly greater amounts of inositol monophosphate (Panel A; P < 0.001), inositol bisphosphate (Panel B; P < 0.001) and inositol triphosphate (Panel C; P < 0.01) following 20 minutes carbachol incubation compared to the distal vaginal strips. Each point represents the mean ± standard deviation (n=4 animals).

Figure 5: A. Representative ethidium bromide gel of mRNA transcripts amplified by RT-PCR utilizing cDNA from human vaginal muscularis and primers specific for muscarinic receptor subtype 2 (M2) and muscarinic receptor subtype 3 (M3). Human bladder and brain were utilized as positive controls. P1: patient 1; P2: patient 2; P3: patient 3. B. Representative tracing of isometric contractile response of the human vaginal muscularis in response to the cumulative addition of carbachol ($10^{-5}$-$10^{-3}$ M). A pEC$_{50}$ of 3.99 was calculated for the contractile force of human vaginal muscularis in response to carbachol.
**Table 1**: Demographic and medical information of patients who consented to participate in the study and from whom vaginal biopsies were obtained for molecular and physiological studies.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Parity</th>
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<td>Utero-vaginal prolapse</td>
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</tbody>
</table>
B

A

M₃

M₉

\[ K\text{Cl} \]

10 \text{ mM}

100 \text{ \mu M}

1 \text{ mM}

Carbachol

1 \text{ \mu M}

10 \text{ \mu M}

100 \text{ \mu M}

1 \text{ mM}

Vaginal Muscles

B1

B2

B3

Bladder

Brain

1 min.

0.5 grams