THE CORRELATION OF VESICULAR TRAFFIC AND TRANSMITTER RELEASE IN BLADDER UROTHELIAL CELLS: INVOLVEMENT OF UROTHELIAL MUSCARINIC RECEPTORS AND OVERACTIVE BLADDER

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Hypothesis / aims of study
Patients with overactive bladder (OAB) are typically treated with muscarinic receptor antagonists. While these agents target muscarinic receptors on the bladder smooth muscle, evidence that the urothelium exhibits “neuron-like” properties and expresses a complement of muscarinic receptors (M1-M5) has sparked an interest in the role of the urothelium in these disorders. Given that antimuscarinic agents effectively enhance the storage phase of micturition, when parasympathetic nerves are silent, it has been postulated that the release of acetylcholine (ACh) from the urothelium may contribute to detrusor overactivity. This study was undertaken to investigate: (1) the expression and function of the non-neuronal cholinergic system in cultured urothelial cells from the rat urinary bladder, (2) the trafficking events underlying transmitter (ACh and ATP) release by these cells and (3) the role of subtype specific muscarinic antagonists in urothelial signaling.

Study design, materials and methods
Urinary bladders were excised from female Sprague-Dawley rats (250-300 g), killed by inhalation of medical grade CO2 followed by thoracotomy and cardiac puncture. The bladders were cut open, gently stretched and pinned with the urothelial side up. Following an overnight incubation in MEM medium containing 2.5 mg/ml dispase, the urothelium was gently scraped from underlying tissue, treated with 0.25% trypsin and following resuspension cells were plated on collagen-coated cover slips. Reverse transcription-PCR: RNA was extracted from cultured cells at the time of confluency (2-3 days in culture) by homogenization in Trizol and reverse transcribed with an oligo-dT primer, using Superscript II (Life technologies). PCR amplification was conducted using primers for the following: rat organic cation transporters, the acetylcholine-synthesizing enzyme choline acetyltransferase (ChAT), the vesicular acetylcholine transporter (VACHT), the high-affinity choline transporter (CHT1) and carnitine acetyltransferase (CarAT).

Acetylcholine and ATP release: Acetylcholine was measured with a radiolabeled method (H3-choline). Urothelial cells were incubated in Krebs solution containing 10-6 M [3H]-choline (specific activity 83.0 Ci mmol-1) for 60 min. The effect of mechanical or chemical stimulation was investigated by changing the superfusate medium to one of a lower osmolarity or containing the drug; samples were taken every minute. Supernasate fractions (100 l) were added to scintillation vials followed by the addition of 4 ml scintillation fluid. At the end of each experiment the cells were lysed and the total volume lysate was collected. This count was added to the cumulative counts from all fractions collected during the course of the experiment to estimate the ‘total releasable’ 3H-ACh. For ATP release, all agents were bath applied and perfusate collected (100 l, 30 s intervals). ATP levels are quantified using a luciferin-luciferase reagent (Sigma) and data normalized with respect to the maximum ATP release following application of the Ca2+ ionophore, A23187 (3 M).

Live cell imaging of vesicular movement using membrane impermeant dyes (FM): We used fluorescent FM dyes as an “activity marker” in order to track the movement of stimulus-evoked dye filled vesicles. This approach is used to provide further information as to the trafficking events underlying transmitter release by urothelial cells. Urothelial cells were incubated in Krebs solution containing the dye FM1-43 (4 M). In the absence of a stimulus, the plasma membrane will fluoresce but the inside of the cell will not. Vesicle recycling will be triggered by either a chemical (ACh or ATP; 10-100 M) or mechanical (hypotonic media) stimulus. This results in an “endocytosis” of labeled plasma membrane whereby the cytoplasm now contains fluorescent vesicles. Following a washout of the cells, the cells are again stimulated with corresponding movement of FM-tagged vesicles to the plasma membrane (“exocytosis”). The loss of fluorescent particles from the cytoplasm provides a measure of exocytosis.

Results
We utilized an FM-dye (FM1-43) to examine the trafficking events (ie vesicular movement) underlying ACh- and ATP-mediated transmitter release in urothelial cells. Using this technique, we first show that ACh elicits a different “fingerprint” pattern of vesicular movement as compared to ATP stimulation. Our data reveals that ATP (10 M) elicits a non-desensitizing “cyclic” pattern of exocytosis and endocytosis (traffic). This pattern was blocked by pretreatment with the purinergic antagonist, suramin (100 M) demonstrating that the effect is mediated by stimulation of urothelial purinergic receptors. In contrast, acetylcholine-induced stimulation elicited a different pattern of regulated vesicle recycling which consisted of a slower but steady increase in cell fluorescence which was significantly lower in magnitude than that seen when the cell was exposed to ATP. In addition, application of the M3-muscarinic muscarinic antagonist 4-DAMP (50-100 nM) alone increased vesicular movement, while the M2-muscarinic antagonist AF6-166 (50-100 nM) alone produced no movement of dye-filled vesicles. Urothelial cells express the acetylcholine synthesizing enzymes, choline acetyltransferase (ChAT) and carnitine acetyltransferase (CarAT). In contrast to neurons, urothelial cells do not express the vesicular acetylcholine transporter (VACHT) but do express OCT3, a subtype of polyspecific organic cation transporter that is thought to be involved in the release of acetylcholine from non-neuronal cells. Though the content of the dye-filled vesicles is not known, experiments measuring both ACh and ATP following mechanical and chemical stimuli were done to correlate transmitter release with vesicular movement in urothelial cells. While mechanical (hypotonic media) elicited a significant release of ACh as compared to control, application of ACh alone did not alter control release. However, following pre-incubation with the nonselective muscarinic receptor antagonist atropine and in its continued presence, ACh evoked an increase in radioactivity release. ACh release was also evoked following application of the M3 muscarinic antagonist, 4-DAMP. These data suggest that ACh may participate in a negative feedback mechanism by acting on muscarinic receptors to inhibit its own release in the urothelium. In addition, pretreatment of urothelial cells with brefeldin, an agent which disrupts vesicular exocytosis, did not block mechanical-evoked ACh release from urothelium but did block ATP release. In contrast to acetylcholine, ATP could be...
released following a mechanical (hypotonic) stimulus as well as following application of ACh (10-100 M). ACh-evoked ATP release was enhanced when applied in the presence of the M3-muscarinic receptor antagonist, 4-DAMP (50-100 nM).

**Interpretation of results**
The location of bladder efferent/afferent nerves in close proximity to the urothelium suggests a paracrine/autocrine regulation of urothelial cells. Stimulation of urothelial muscarinic receptors elicits movement of dye-filled vesicles, and this type of “traffic” is increased in the presence of a M3-muscarinic antagonist. Given that this type of stimulation also elicits a significant release of ATP from urothelium, it is likely that stimulation of membrane “traffic” involves release of ATP. In addition, the lack of effect of brefeldin on mechanically-evoked ACh release as compared to ATP release suggests that urothelial-derived ACh release involves a non-vesicular mechanism.

**Concluding message**
We conclude from these studies that (1) agents (brefeldin) that disrupt vesicular exocytosis decreases ATP but not ACh release from urothelium. Thus other agents such as botulinum toxin that also block exocytosis and are used to treat OAB symptoms may not act by suppression of urothelial ACh release and (2) Blockade of urothelial M3-muscarinic receptors leads to additional release of transmitters such as ATP and to a lesser extent ACh, suggesting that M2-muscarinic receptor selective antagonists may be more effective in the treatment of bladder disorders including OAB.

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**ANIMAL SUBJECTS:** This study followed the guidelines for care and use of laboratory animals and was approved by University of Pittsburgh Ethics Committee