Nerve-released acetylcholine contracts urinary bladder smooth muscle by inducing action potentials independently of IP3-mediated calcium release

Bernhard Nausch, Thomas J. Heppner, and Mark T. Nelson
Department of Pharmacology, University of Vermont, Burlington, Vermont
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Nausch B, Heppner TJ, Nelson MT. Nerve-released acetylcholine contracts urinary bladder smooth muscle by inducing action potentials independently of IP3-mediated calcium release. Am J Physiol Regul Integr Comp Physiol 299: R878–R888, 2010. First published June 23, 2010; doi:10.1152/ajpregu.00180.2010.—Nerve-released ACh is the main stimulus for contraction of urinary bladder smooth muscle (UBSM). Here, the mechanisms by which ACh contracts UBSM are explored by determining Ca2+ waves within the smooth muscle that were present only during stimulus application. Ca2+ waves were blocked by inhibition of muscarinic ACh receptors (mAChRs) with atropine and depletion of sarcoplasmic reticulum Ca2+ stores with cyclopiazonic acid (CPA), and therefore likely reflect activation of IP3 receptors (IP3Rs). Electrical field stimulation also increased excitability to induce action potentials (APs) that were accompanied by Ca2+ flashes, reflecting Ca2+ entry through voltage-dependent Ca2+ channels (VDCCs) during the action potential. The evoked Ca2+ flashes and APs occurred as a burst with a lag time of ~1.5 s after onset of stimulation. They were not inhibited by blocking IP3-mediated Ca2+ waves, but by blockers of mAChRs (atropine) and VDCCs (diltiazem). Nerve-evoked contractions of UBSM strips were greatly reduced by blocking VDCCs, but not by preventing IP3-mediated Ca2+ signaling with cyclopiazonic acid or inhibition of PLC with U73122. These results indicate that ACh released from nerve varicosities induces IP3-mediated Ca2+ waves during stimulation; but contrary to expectations, these signals do not appear to participate in contraction. In addition, our data provide compelling evidence that UBSM contractions evoked by nerve-released ACh depend on increased excitability and the resultant Ca2+ entry through VDCCs during APs.

muscarnic receptor; sarcoplasmic reticulum; IP3 receptor; voltage-dependent calcium channel

THE URINARY BLADDER SERVES TWO FUNCTIONS: STORAGE AND VOIDING OF URINE. Micturition (the elimination of urine) is triggered by the action of ATP and ACh, which are released from parasympathetic nerves, on purinergic receptors (P2XRs) and muscarinic ACh receptors (mAChRs), respectively (2, 20, 44). The relative contribution of each pathway to contraction depends on stimulation frequency and varies by species, but the muscarinic pathway is a major mediator of micturition in all species (2). In humans, mAChRs predominantly mediate micturition under normal conditions, but purinergic mechanisms become prominent under pathological conditions, such as overactive bladder (33, 38) or interstitial cystitis (34).

The prevailing view is that ACh contracts urinary bladder smooth muscle (UBSM) by inositol 1,4,5-trisphosphate (IP3)-mediated Ca2+ release from the sarcoplasmic reticulum (SR) through activation of M3 mAChRs (3, 12). M3 mAChRs canonically couple to the G protein Gq, which activates PLC to produce IP3 (8). Stimulation of M3 mAChRs has been shown to activate IP3 production (32, 40) and to promote contraction (26, 28). IP3-mediated release of Ca2+ from the SR has been demonstrated by photolysis of caged IP3 in isolated colonic smooth muscle cells (29). However, a causal link between IP3-mediated Ca2+ release and nerve-evoked contraction under physiological conditions has not been established in UBSM.

On the other hand, there is evidence that an increase in excitability and consequent Ca2+ influx through voltage-dependent Ca2+ channels (VDCCs) during action potentials (APs) are important for contraction. Creed et al. (5) have shown that ACh increases AP frequency in rabbit UBSM. In guinea pig, nerve-stimulation (with purinergic receptors disabled) causes a transient increase in AP frequency (14). In accord with these data, disabling VDCCs greatly reduces contractile force induced by muscarinic agonists (43, 47) or nerve stimulation (21). Hence, the mechanisms by which mAChRs increase intracellular Ca2+ to subsequently cause contraction are not fully resolved.

In the present study, we investigated the elementary signaling events evoked by nerve-released ACh and their contribution to contraction. Because bath application of muscarinic agonists lacks the spatial and temporal aspects of normal bladder physiology, we used electrical field stimulation (EFS) to evoke release of ACh from parasympathetic nerve fibers in UBSM strips and thereby physiological delivery of ACh to the smooth muscle cells. High-speed confocal Ca2+ imaging was used to investigate the Ca2+ waves evoked by nerve-released ACh and revealed two primary Ca2+ events: 1) Ca2+ waves, reflecting IP3-mediated Ca2+ release from the SR, and 2) Ca2+ flashes, reflecting Ca2+ influx through VDCCs during APs. These two events can be distinguished by their temporal, spatial, and pharmacological properties. In addition, increases in UBSM excitability and AP frequency in response to nerve-released ACh were determined directly by measuring membrane potential (Vm). Contrary to prevailing assumptions, our results do not support an essential role for IP3-mediated Ca2+ signaling in nerve-evoked UBSM contractility. Instead, our results indicate that increased UBSM excitability, measured as Vm depolarization, APs, and Ca2+ flashes, plays a central role in mediating the contractions induced by nerve-released ACh.

MATERIALS AND METHODS

Solutions. All experiments were performed in physiological saline solution (PSS in mM: 119 NaCl, 4.7 KCl, 24 NaHCO3, 1.2 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, 11 glucose, and 23 μM EDTA). HEPES buffered solution (HBS in mM: 134 NaCl, 6 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 glucose, pH adjusted to 7.3 with NaOH) was used...
for tissue dissection and loading of tissues with fluo-4 and caged IP$_3$ [iso-Ins (1,4,5)P$_3$-PM (iso-IP$_3$)].

**Animals and tissue.** All procedures were approved by the Institutional Animal Care and Use Committee of the University of Vermont. Male C57BL/6J mice (3 to 4 mo of age) were used for all experiments. Mice were euthanized by intraperitoneal injection of pentobarbital sodium followed by decapitation. The abdominal cavity was opened by a midline incision, and the urinary bladder was quickly removed and transferred to ice-cold HBS. The bladder was then cut open by a longitudinal incision, and residual urine was rinsed away with ice-cold HBS. Detrusor strips obtained from the area above the ureteric orifices and dissected free of mucosa were used in all experiments.

**Spinning disc confocal microscopy.** Detrusor strips (~1 × 4 mm) with only a few layers of muscle bundles were dissected from the serosal side of the bladder, pinned onto blocks made from Syilgard (Dow Corning, Midland, MI), and loaded with the fluorescent Ca$^{2+}$ indicator fluo-4 AM (10 μM; Molecular Probes, Eugene, OR) for 60 to 90 min at 30°C in HBS. For experiments involving photolysis of caged IP$_3$, the tissue was also loaded with 10 μM iso-IP$_3$ (Axon, San Diego, CA). Fluromer F-127 (0.04%; Invitrogen; Thermo Fisher, CA) was added to facilitate loading. After washing for at least 20 min in HBS, detrusor strips were placed in a tissue chamber, superfused with PSS (3 ml/min, 35–37°C, bubbled with 95% O$_2$ and 5% CO$_2$) containing 10 μM α,β-methylene ATP (α,β-meATP) to desensitize purinergic receptors, and allowed to equilibrate for 20 min. Fluo-4 was excited by a krypton-argon laser at 488 nm, and emission above 512 nm was recorded with a charge-coupled device camera (model XR Mega10; Stanford Photonics, Palo Alto, CA) coupled to an upright microscope (model EM600FN; Nikon, Japan) equipped with a QCL 100 scanhead (Solamen, Salt Lake City, UT) and a ×60 water-immersion objective (numerical aperture 1.0; Nikon). Images of 512 × 512 pixels (119 × 119 μm) were acquired at a rate of 15 or 60 images/s using In Vivo software (Media Cybernetics, Bethesda, MD). Ca$^{2+}$ events were evoked by EFS or photolysis of iso-IP$_3$, EFS (0.2-ms pulse duration, 20 Hz, single train of 1-s duration) was generated by an S-44 stimulator (Grass Technologies, West Warwick, RI) and delivered to the tissue via two platinum wires parallel to the detrusor strip. A UV flash (~1 ms duration) from a xenon arc lamp-based flash unit (Rapp OptoElectronics, Hamburg, Germany) coupled to a custom-made condenser (Till Photonics, Gräfeling, Germany) was used for photolysis of iso-IP$_3$ throughout the entire field of view. Concentrated stock solutions of atropine, diltiazem, and cyclopiazonic acid (CPA), used as indicated in the text, were added to the superfusing PSS. The number and lag time of EFS-induced Ca$^{2+}$ events, as well as the maximum increase in fractional fluorescence intensity (∆F/ F$_0$) of events caused by photolysis of iso-IP$_3$ were analyzed with custom-written software (SparkAn; Dr. Adrian Bonev, Nelson Laboratory, University of Vermont, Burlington, VT). An average fluorescence value obtained from 10 images immediately prior to stimulation was used as F$_0$. Strips that did not respond to EFS after application of CPA or diltiazem and those in which excessive tissue movement prevented accurate quantification of Ca$^{2+}$ events were excluded from the analysis.

**Intracellular recording.** Small strips (~1 × 2 mm) containing a single layer of smooth muscle bundles were carefully dissected from the serosal side of the bladder and pinned onto the bottom of a recording chamber. The tissue was permanently superfused with PSS (3 ml/min, 35–37°C, bubbled with 95% O$_2$ and 5% CO$_2$) containing 10 μM α,β-meATP. After superfusing for ~30 min, individual smooth muscle cells were impaled with KCl (0.5 M)-filled sharp microelectrodes having a resistance of ~225 MΩ. APs were evoked by EFS (0.2-ms pulse duration, 20 Hz, 1-s train duration), and the tissue was stimulated twice before (control) and 15 min after application of drugs. Atropine, CPA, and diltiazem were added as concentrated stock solutions to the superfusing PSS.

Data were acquired at a rate of 2 or 20 kHz, and the signal was amplified with an Axoclamp 2A amplifier (MDS Analytical Technologies, Sunnyvale, CA). pClamp software (MDS Analytical Technologies, Jolla, CA) was used to record the data and analyze the time from the onset of EFS to each individual AP as well as the number of APs in a burst following EFS. To determine Vm changes in response to EFS, V_m during Ca$^{2+}$ wave activity (average of four time points between 250 and 450 ms after onset of EFS) or during Ca$^{2+}$ flash activity (average of four time points between 2 and 2.5 s after onset of EFS) was compared with V_m before stimulation.

**Myography.** After removal of the bladder base and mucosa, the remaining tissue was cut into eight strips (~1.5 × 6 mm) oriented from the dome to the base of the bladder. A loop of silk suture (5-0) was attached to either end of the strip, and the tissue was transferred to the organ bath of a myograph system (Med Associates, Georgia, VT) filled with PSS (37°C, bubbled with 95% O$_2$:5% CO$_2$). One end of the strip was attached to a force transducer, and contractile force was recorded using MED Myograph software (Med Associates, St. Albans, VT) initially, a tension of 10 mN was applied, and the tissue was allowed to equilibrate for 1 h. During equilibration, PSS was changed every 20 min; after equilibration, α,β-meATP (10 μM) was added to facilitate loading. After a 20-min incubation period, contractions were evoked by EFS generated by a PHM-152V stimulator and delivered to the tissue via platinum electrodes parallel to either side of the detrusor strip. Frequency response curves were obtained by applying trains of pulses (0.2-ms pulse duration, 20 V, 2-s train duration) every 3 min with increasing frequency (0.5, 2, 3.5, 5, 7.5, 10, 12.5, 15, 20, 30, 40, 50, and 75 Hz). Atropine, diltiazem, CPA, and U73122 were added directly to the bath as concentrated stock solutions 5 min after the first frequency response curve and a second frequency response curve was obtained after incubating for 20 min. Force amplitude, force integral, and half-amplitude duration, as well as the lag time of peak force of EFS-evoked contractions were analyzed using MyoViewer (Med Associates) and MiniAnalysis (Synaptosoft, Fort Lee, NJ) software. Force integral was measured as area under the curve from the onset of contraction to 95% relaxation.

**Chemicals and reagents.** Fluo-4 and Fluronic F-127 were purchased from Molecular Probes (Eugene, OR), iso-IP$_3$ from Axxora (San Diego, CA), and U73122 and CPA from Calbiochem (San Diego, CA). All other chemicals and reagents were obtained from Sigma (St. Louis, MO). Stock solutions of CPA, fluo-4, and iso-IP$_3$ were prepared in DMSO; α,β-meATP and diltiazem were prepared in water. U73122 was dissolved in chloroform, aliquoted, desiccated under argon, and reconstituted in DMSO prior to each experiment.

**Analysis and statistics.** Prism software (GraphPad Software, La Jolla, CA) was used for statistical tests and preparation of graphs. Data are expressed as means ± SE unless otherwise noted. Student’s t-test and two-way repeated-measures ANOVA followed by Bonferroni post tests were used for comparison of two or more groups, as appropriate, and P < 0.05 was considered statistically significant. Unless stated otherwise, the number of experiments (n) refers to the number of tissue strips.

**RESULTS**

**Photolysis of caged IP$_3$ releases Ca$^{2+}$ from SR Ca$^{2+}$ stores, but does not induce Ca$^{2+}$ flashes.** Stimulation of M3 mACHRs activates PLC to produce IP$_3$ (23, 32, 40), and IP$_3$, released by photolysis of caged IP$_3$, acts on IP$_3$Rs to release Ca$^{2+}$ from the SR in isolated colonic smooth muscle cells (29). Using a similar approach to study IP$_3$-mediated Ca$^{2+}$ release in intact strips of UBSM, we found that photolysis of iso-IP$_3$ by a short pulse of UV light (~1-ms duration) significantly increases intracellular Ca$^{2+}$ (∆F/ F$_0$: 1.10 ± 0.34, mean ± SD; Fig. 1, A–D). Exposure to UV did not elicit any measurable response in tissue strips not loaded with iso-IP$_3$ (∆F/ F$_0$: 0.01 ± 0.01, mean ± SD; Fig. 1D), indicating that the increase in Ca$^{2+}$ was due to photolysis of iso-IP$_3$. The response to IP$_3$ photolysis
not distinguish between these two possibilities, we selectively eliminated the SR Ca\textsuperscript{2+} stores by cyclopiazonic acid (CPA) prevented IP\textsubscript{3}-induced Ca\textsuperscript{2+} release. Inhibition of voltage-dependent Ca\textsuperscript{2+} channels (VDCCs) by diltiazem for 15 min had no effect. Significance by paired t-test; data are means ± SE.

Our results indicate that nerve-released ACh induces Ca\textsuperscript{2+} waves during stimulation that are supplant by Ca\textsuperscript{2+} flashes after stimulation ceases. The inability to detect Ca\textsuperscript{2+} waves after Ca\textsuperscript{2+} flash activity begins could mean that Ca\textsuperscript{2+} waves actually cease after the stimulation period has ended; alternatively, Ca\textsuperscript{2+} waves may continue to occur but are obscured by Ca\textsuperscript{2+} flashes. To distinguish between these two possibilities, we selectively eliminated Ca\textsuperscript{2+} flashes by inhibiting VDCCs with diltiazem. In the presence of diltiazem, EFS-induced Ca\textsuperscript{2+} waves abruptly ceased with cessation of stimulation (Fig. 2G), indicating that Ca\textsuperscript{2+} waves persist only as long as EFS is applied.

The rapid induction of atropine-sensitive Ca\textsuperscript{2+} waves following the onset of EFS suggests that IP\textsubscript{3} levels rise rapidly to stimulate IP\textsubscript{3}-R-mediated release of SR Ca\textsuperscript{2+}. In support of this, prevention of SR Ca\textsuperscript{2+} uptake with the SERCA inhibitor CPA (10 \mu M) eliminated Ca\textsuperscript{2+} waves (4.2 ± 1.3 in control vs. 0.2 ± 0.2 after CPA; Fig. 2H). In contrast, inhibition of VDCCs with diltiazem (50 \mu M) did not prevent Ca\textsuperscript{2+} waves (5.3 ± 0.8 in control vs. 4.6 ± 0.5 after diltiazem; Fig. 2H), consistent with the interpretation that Ca\textsuperscript{2+} waves reflect IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release from the SR. On the other hand, Ca\textsuperscript{2+} flashes were
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Fig. 2. Stimulation of muscarinic receptors causes Ca\textsuperscript{2+} waves followed by Ca\textsuperscript{2+} flashes. A: fluo-4 loaded UBSM strip with ROIs (colored boxes) in which F/F\textsubscript{0} (B and C) was measured. The line scan (D) was performed offline along the yellow line. B: representative recording of F/F\textsubscript{0} during a Ca\textsuperscript{2+} wave. Note the sequential rise in F/F\textsubscript{0} in the 3 ROIs indicating propagation. C: representative recording of F/F\textsubscript{0} during Ca\textsuperscript{2+} flashes. Note the simultaneous rise in F/F\textsubscript{0} in all three ROIs. D: line scan showing the different Ca\textsuperscript{2+} events evoked by nerve-released ACh. Ca\textsuperscript{2+} waves were characterized by propagation (sloped line), whereas Ca\textsuperscript{2+} flashes followed a burst \(\sim 1–6\) s after the onset of stimulation \((n = 145\) waves and 521 flashes from 21 strips). G: blocking Ca\textsuperscript{2+} flashes with diltiazem (50 \(\mu\)M) revealed that Ca\textsuperscript{2+} wave activity stops with cessation of stimulation \((n = 24\) waves from 7 strips). H: Ca\textsuperscript{2+} waves were sensitive to atropine (10 \(\mu\)M) and CPA (10 \(\mu\)M), but not to diltiazem (50 \(\mu\)M). I: Ca\textsuperscript{2+} flashes were blocked by atropine and diltiazem, but not by CPA. Significance by paired t-test; data are means ± SE.

completely prevented by application of diltiazem (26.8 ± 5.3 in control vs. 0 ± 0 after diltiazem; Fig. 2F), as we have shown previously (19), but depletion of SR Ca\textsuperscript{2+} with CPA did not reduce the number of Ca\textsuperscript{2+} flashes (44.4 ± 13.9 in control vs. 29.8 ± 8.3 after CPA; Fig. 2F), even in the absence of purinergic receptor inhibitors \((P = 0.3850\); paired t-test, \(n = 5\)). Combined application of diltiazem and CPA abolished all Ca\textsuperscript{2+} events \((P = 0.0130\) for Ca\textsuperscript{2+} waves, \(P = 0.0243\) for Ca\textsuperscript{2+} flashes; paired t-test, \(n = 6\)).

Collectively, these data show that, with purinergic receptors desensitized, nerve stimulation by EFS evokes rapid Ca\textsuperscript{2+} release from SR Ca\textsuperscript{2+} stores, which manifests as Ca\textsuperscript{2+} waves, and Ca\textsuperscript{2+} influx through VDCCs in the form of Ca\textsuperscript{2+} flashes. These two events occur independently of each other since CPA did not abolish Ca\textsuperscript{2+} flashes and diltiazem did not affect the number of Ca\textsuperscript{2+} waves. Accordingly, CPA can be used to disable SR Ca\textsuperscript{2+} release without inhibiting Ca\textsuperscript{2+} entry, and diltiazem to selectively block Ca\textsuperscript{2+} influx.

Activation of mACHr by nerve-released ACh elicits a burst of APs. To explore the involvement of SR Ca\textsuperscript{2+} release in neurally mediated increases in excitability, intracellular recording techniques were used to determine the electrical response of UBSM strips to EFS. The \(V_m\) recorded in individual smooth muscle cells in UBSM strips was \(-43 ± 5\) mV \((\text{mean} ± \text{SD}, \text{n} = 26), \) a value similar to that previously reported for mouse UBSM (20, 30), and few cells exhibited spontaneous APs. In the presence of \(\alpha,\beta\)-meATP \((10 \mu\text{M})\) to block purinergic signaling, EFS \((20\) Hz, 1-s train duration) caused a burst of APs (Fig. 3A), with the first AP occurring 1.2 ± 0.5 s after the beginning of EFS \((\text{mean} ± \text{SD}, \text{n} = 36).\) Each burst consisted of 10.3 ± 6.0 APs and had a duration of 3.4 ± 2.0 s \((\text{mean} ± \text{SD}, \text{n} = 36).\) The number of APs evoked by EFS did not change significantly over time \((11.4 ± 3.0\) vs. 14.8 ± 5.3 initially and after a resting period of 15 min, respectively; \(P = 0.2434\); paired t-test, \(n = 4\)). Atropine greatly reduced the number of APs triggered by EFS \((7.3 ± 1.1\) in control vs. 0.8 ± 0.5 after atropine; Fig. 3, D and G), confirming that the burst of APs was due to activation of mACHRs. The upstroke of APs in UBSM is caused by Ca\textsuperscript{2+} influx through VDCCs (14, 15, 18, 19); thus, as expected, application of 50 \(\mu\)M diltiazem completely abolished APs \((10.2 ± 2.0\) APs in control vs. 0 ± 0 after diltiazem; Fig. 3, E and G).

Because IP\textsubscript{3}-mediated Ca\textsuperscript{2+} waves precede Ca\textsuperscript{2+} flashes/APs (Fig. 2, E and F), it is conceivable that SR Ca\textsuperscript{2+} release could modulate excitability and affect nerve-evoked APs. However, blocking Ca\textsuperscript{2+} waves with CPA did not affect the induction of Ca\textsuperscript{2+} flashes (Fig. 2J), suggesting that Ca\textsuperscript{2+} waves neither suppress APs during stimulation nor trigger them following stimulation. Similarly, depletion of SR Ca\textsuperscript{2+} stores with the SERCA blocker CPA (10 \(\mu\)M) did not inhibit APs \((12.0 ± 1.4\) APs in control vs. 11.7 ± 2.4 after CPA; Fig. 3, F and G), indicating that release of Ca\textsuperscript{2+} from the SR is not involved in temporal aspects of increased excitability. Consistent with this, Ca\textsuperscript{2+} waves appearing during the 1-s stimulation interval did not cause membrane depolarization, but instead were associated with a small, but significant, hyperpolarization \((\Delta V_m: -1.8 ± 0.7\) mV, \(P = 0.0325\) compared with the hypothetical mean 0, \text{n} = 15) that was absent after depletion of SR Ca\textsuperscript{2+} waves.
stores with CPA (ΔV_m: 0.2 ± 0.3 mV, P = 0.5707 compared with the hypothetical mean 0, n = 6). This lends further support to the hypothesis that IP3-mediated Ca2+ release does not increase excitability.

Similar to Ca2+ flashes, 50% of observed APs occurred between 1.9 and 3.8 s after onset of EFS (25th and 75th percentile, respectively; Fig. 3, B and C). Interestingly, during this interval (2 to 2.5 s after onset of EFS), we observed a small, but statistically significant depolarization in the presence of diltiazem (ΔV_m: 2.4 ± 0.9 mV, P = 0.0420 compared with the hypothetical mean 0, n = 6; Fig. 3H), but not after pretreatment with the specific muscarinic antagonist atropine (ΔV_m: –0.5 ± 0.3 mV, P = 0.1531 compared with the hypothetical mean 0, n = 5; Fig. 3H). These data suggest that nerve-released ACh induces a delayed depolarization, which could contribute to the increase in action potential frequency.

Ca2+ influx, but not IP3-mediated Ca2+ release, is essential for contraction. Previously, we have shown that the combined block of muscarinic and purinergic receptors completely abrogates nerve-evoked contractions in mouse UBSM, and inhibition of mAChRs alone is sufficient to eliminate nerve-evoked contractions in the absence of P2XRs (20), demonstrating that mouse urinary bladder contraction is mediated exclusively by purinergic and muscarinic receptors. Here, we found that, after inhibition of purinergic receptors with α,β-meATP (10 μM), EFS caused frequency-dependent contractions ranging from 2.57 ± 1.46 mN·s at a stimulation frequency of 0.5 Hz to 151.51 ± 47.12 mN·s at 75 Hz (force integral, mean ± SD, n = 41). Contractions evoked by 20 Hz stimulation reached peak force 2.75 ± 0.32 s after initiating EFS (mean ± SD, n = 56), and half-amplitude duration was 5.7 ± 1.1 s (mean ± SD, n = 41). Pretreatment with the specific muscarinic antagonist atropine (10 μM) almost completely abolished the contractile response (Fig. 4A); it significantly reduced force amplitude at stimulation frequencies ≥ 5 Hz (Fig. 4, A, d), force integral ≥ 7.5 Hz (Fig. 4, A, e), and half-amplitude duration at all stimulation frequencies (Fig. 4, A, f).

To investigate the roles of Ca2+ waves and Ca2+ flashes in nerve-evoked contractions of UBSM, we used CPA (10 μM) and diltiazem (50 μM), which can be used to selectively inhibit Ca2+ waves and Ca2+ flashes/APs, respectively, as shown by imaging and electrical data (Figs. 1–3). Blocking VDCCs significantly inhibited contractions evoked by nerve-released ACh (Fig. 4B), reducing force amplitude at stimulation frequencies ≥ 3.5 Hz (Fig. 4, B, d), force integral ≥ 5 Hz (Fig. 4, B, e), and half-amplitude duration at all stimulation frequencies (Fig. 4, B, f). In contrast, inhibition of SR Ca2+ release by blocking SERCA with CPA did not reduce contractile force (Fig. 5A); instead, it increased force integral at stimulation frequencies ≥ 12.5 Hz (Fig. 5, A, e) due to an increase in half-amplitude duration (Fig. 5, A, f) without any change in force amplitude (Fig. 5, A, d). Combined application of CPA...
and diltiazem reduced force amplitude significantly at stimulation frequencies ≥ 3.5 Hz, force integral ≥ 5 Hz, and half-amplitude duration at all stimulation frequencies (P < 0.05, two-way repeated-measures ANOVA; n = 7), but the effects on force amplitude and integral were not significantly different from that obtained with diltiazem alone (Fig. 5B). These data indicate that SR Ca^{2+} release is not required for the elevation of force evoked by nerve-released ACh, but do not strictly preclude a functional role for IP3. To further examine this possibility, contractile responses were measured in the presence of the PLC inhibitor U73122 (Fig. 5C), which has been shown to prevent mAChR-induced increases in IP3 (37). U73122 (10 μM) augmented force amplitude slightly at stimulation frequencies of 2, 3.5, and 5 Hz (Fig. 5, C, d). At stimulation frequencies ≥ 20 Hz, U73122 reduced force integral (Fig. 5, C, e) and decreased half-amplitude duration at stimulation frequencies ≥ 12.5 Hz (Fig. 5, C, f). Although statistically significant, the maximum reduction of the force integral in the presence of U73122 was only 14.6 ± 4.8% (at 30 Hz stimulation frequency, n = 7). The minimal effects of the PLC inhibitor U73122 also support the concept that IP3-mediated Ca^{2+} release does not have a major role in neurally evoked contractions of UBSM.

DISCUSSION

Micturition depends on a forceful and coordinated contraction of UBSM that is brought about by activation of muscarinic receptor stimulation. A: representative recordings of contractions evoked by EFS with increasing frequency in the presence of α,β-methylene ATP (10 μM) before (A, a) and after (A, b) application of atropine (10 μM). Contractions in response to 20 Hz stimulation are shown on a faster time scale (A, c). Atropine significantly reduced force amplitude (A, d), force integral (A, e), and half-amplitude duration (A, f). B: representative recordings of nerve-evoked contractions in the presence of α,β-methylene ATP (10 μM) before (B, a) and after (B, b) application of diltiazem (50 μM). Contractions in response to 20 Hz stimulation are shown on a faster time scale (B, c). Diltiazem significantly reduced force amplitude (B, d), force integral (B, e), and half-amplitude duration (B, f). *P < 0.05 and ***P < 0.001 by two-way repeated-measures ANOVA; data are means ± SE.
Fig. 5. SR Ca\(^{2+}\) release does not promote contractility. A: representative recordings of nerve-evoked contractions in the presence of α,β-meATP (10 μM) before (A, a) and after (A, b) application of CPA (10 μM). Contractions in response to 20 Hz stimulation are shown on a faster time scale (A, c). CPA had no effect on force amplitude (A, d), but significantly increased force integral (A, e) and half-amplitude duration (A, f).

B: contractions evoked by 20 Hz stimulation in the presence of diltiazem alone and diltiazem with CPA (B, a). Combined inhibition of SR Ca\(^{2+}\) release and Ca\(^{2+}\) influx was not different from blocking only Ca\(^{2+}\) influx. Note that the graphs for force amplitude (B, b) and force integral (B, c) coincide (P > 0.05 by two-way ANOVA).

C: representative recordings of nerve-evoked contractions in the presence of α,β-meATP (10 μM) before (C, a) and after (C, b) application of U73122 (10 μM). Contractions in response to 20 Hz stimulation are shown on a faster time scale (C, c). U73122 slightly increased force amplitude at 2, 3.5, and 5 Hz stimulation frequency (C, d), but reduced force integral by maximally ~15% (C, e) and half-amplitude duration (C, f) at stimulation frequencies > 20 Hz and 12.5 Hz, respectively. *P < 0.05, **P < 0.01 and ***P < 0.001 by two-way repeated-measures ANOVA; data are means ± SE.
Muscarinic paradigm. UBSM expresses two types of mAChRs: M2 and M3 (17). Although the M2 subtype is more abundant, M3 mAChRs mediate ACh-dependent contraction, as evidenced by studies employing selective inhibitors of mAChR subtypes (9, 16, 25, 26) and mAChR knockout mice (28, 39). Canonicallly, M3 mAChRs signal through the G protein Gαq, which activates PLC-mediated IP3 production and thereby induces IP3-mediated release of Ca2+ from the SR (8). Because contraction is directly dependent on cytosolic Ca2+ (6, 27), this cascade has traditionally been presumed to constitute a major signaling pathway to cause contraction (3, 12). It has been shown that stimulation of UBSM M3 mAChRs by muscarinic agonists increases IP3 production (40) and causes contraction (28, 39), and Ji et al. (24) reported EFS-evoked atropine-sensitive Ca2+ waves in intact UBSM strips. Here, we show that photolysis of iso-IP3 increases intracellular Ca2+ in intact UBSM strips. This response was prevented by depletion of SR Ca2+ stores by CPA. In addition, we show that stimulation of mAChRs by nerve-released ACh induces CPA-sensitive Ca2+ waves. Although it is well established that mAChRs evoke IP3R-mediated Ca2+ waves, a causal relationship between this event and contraction has not been established.

PLC, IP3, and IP3-mediated Ca2+ release. IP3-R-mediated Ca2+ wave activity, induced by stimulation of mAChRs by nerve-mediated release of ACh, was only present during EFS and stopped with cessation of stimulus application. This suggests a rapid termination of ACh-dependent signaling through the Gαq/PLC/IP3 pathway, possibly reflecting rapid degradation of IP3. In contrast, contractions evoked by nerve-released ACh reached peak force after cessation of EFS, and their half-amplitude duration was approximately three times longer than the stimulus duration. This suggests that IP3-mediated Ca2+ signals are too short-lived to sustain contractions after cessation of stimulus application.

Although contraction is initiated by cytosolic Ca2+, it depends on phosphorylation of the regulatory myosin light chain (RLC), which is determined by the balance of myosin light chain kinase (MLCK) and phosphatase (MLCP) activities. MLCP activity can be dynamically regulated by the Rho kinase and PKC pathways, and as such can lead to maintained force with decaying Ca2+ (Ca2+ sensitization). Recently, Ding et al. (6) provided evidence that contraction of mouse UBSM strips to EFS (50 Hz, 3-s train duration) is strictly dependent on Ca2+, MLCK activation, and RLC phosphorylation without any apparent involvement of the Rho kinase or PKC pathway. Therefore, IP3-mediated Ca2+ release does not appear capable of initiating and sustaining contractions evoked by nerve-released ACh.

Depleting the SR of Ca2+ by blocking SERCA abolished Ca2+ waves, but did not decrease contractile force. On the contrary, it increased contractile force, possibly by slowing the rate of Ca2+ removal from the cytosol or by preventing Ca2+ sparks and thereby reducing large-conductance Ca2+-activated K+ channel activity (22). Thus, our data do not support an essential role for SR Ca2+ stores in nerve-evoked contraction in UBSM. This conclusion is in accord with several studies that did not find an inhibitory effect of CPA on contractions in urinary bladder (31, 35, 50). The failure of CPA to inhibit contractile force excludes a significant role for SR in providing Ca2+ for contraction.

![Diagram of proposed muscarinic signaling](image-url)
The products of PLC-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), IP_3, and diacrglycerol (DAG), could cause contraction directly by increasing UBSM excitability. For example, IP_3 has been shown to activate canonical transient receptor potential (TRP)δ channels independently of SR Ca^{2+} release in cerebral arteries (48), and DAG could increase excitability through PKC-dependent inhibition of potassium channels (e.g., ATP-sensitive potassium channels) (4) or activation of TRP channels (41). In addition, IP_3 has been shown to inhibit TRP melastatin channels (49) and to activate large-conductance Ca^{2+}-activated K^{+} channels (42). Accordingly, depletion of PIP_2 could increase excitability. The common element in these mechanisms involving IP_3, DAG, and depletion of PIP_2 is PLC-mediated hydrolysis of PIP_2. However, the PLC inhibitor U73122 had little effect on nerve-evoked contractions. This observation is in agreement with the findings of Schneider et al. (37) who showed that U73122 completely blocks carbachol-induced accumulation of inositol phosphates, but does not inhibit carbachol-induced contractions. Our data and those of others (11) do not support a major role of PLC in muscarinic-induced increases in excitability or contractility to nerve stimulation or bath-applied agonists. The role of PKC in muscarinic-induced contractions also appears to be minimal (11). Taken together, these data provide solid evidence that IP_3-mediated Ca^{2+} release does not directly contribute to contractions evoked by nerve-released ACh.

Although muscarinic receptor activation stimulates PLC and increases IP_3-mediated Ca^{2+} waves, their functional role in UBSM remains unclear. Ca^{2+} waves do not appear to trigger APs and Ca^{2+} flashes, because photolysis of caged IP_3 did not induce flashes, and blocking waves with CPA did not affect nerve-evoked AP or Ca^{2+} flashes. It is, however, possible that IP_3-mediated Ca^{2+} waves are responsible for the small CPA-sensitive membrane hyperpolarization that we observed. We have recently reported that purinergic signaling during stimulation suppresses the subsequent, cholinergic-driven Ca^{2+} flashes (20). It is possible that during stimulation, the IP_3-mediated Ca^{2+} waves, in conjunction with purinergic-induced increases in excitability, contribute to suppression of cholinergic-mediated delayed Ca^{2+} flashes. The PLC pathway could also affect excitability under conditions when other target ion channels are active, e.g., the K_{ATP} channel. Longer-term processes, such as activation of Ca^{2+}-dependent transcription factors, could also be a target for IP_3-mediated Ca^{2+} release. For example, nuclear factor of activated T-cells activation as well as proliferation of smooth muscle have been shown to be dependent on IP_3-mediated Ca^{2+} release (13, 45). This area warrants further investigation.

**Excitability and Ca^{2+} influx through VDCCs.** The main trigger for contraction is intracellular Ca^{2+} (2, 6, 27). Here, we provide direct evidence that stimulation of mAChRs increases cytosolic Ca^{2+} by two different mechanisms: 1) IP_3-mediated Ca^{2+} release from the SR, manifested as Ca^{2+} waves, and 2) increased excitability and associated opening of VDCCs, which gives rise to Ca^{2+} flashes and APs. IP_3-mediated Ca^{2+} release was not crucial for contraction; instead, we found that inhibition of VDCCs by diltiazem, which abolished Ca^{2+} flashes and APs, greatly reduced contractile force mediated by nerve-released ACh. In rabbit UBSM, ACh has been shown to increase AP frequency (5), and EFS (with P2X receptors blocked) causes an increase in AP frequency in guinea pig UBSM (14). In addition, we have shown previously that diltiazem inhibits nerve-evoked contractions when both purinergic and muscarinic receptors are available (21). The residual increase in force observed in diltiazem and CPA may reflect a contraction in response to a small, and therefore undetected, Ca^{2+} signal [e.g., PKC-dependent opening of TRP channels (7)] or Ca^{2+}-independent phosphorylation of MLC by Rho kinase, integrin-linked kinase, or zipper-interacting protein kinase (36). Our results, taken together with these previous observations, suggest that mAChRs increase excitability to trigger APs and that Ca^{2+} influx during the AP provides Ca^{2+} for contraction.

Wu et al. (46) have proposed that Ca^{2+} influx through VDCCs contributes to filling of SR Ca^{2+} stores. It has also been suggested that stimulation of mAChRs leads to Ca^{2+} influx through VDCCs, followed by uptake of Ca^{2+} into the SR and subsequent IP_3-mediated release from the SR to cause contraction (35). However, our data do not support this model. First, in the present study, inhibition of VDCCs reduced neither SR Ca^{2+} release in response to photolysis of caged IP_3 nor the frequency of Ca^{2+} waves evoked by EFS. Secondly, CPA would be expected to have an effect on contraction similar to that of diltiazem, because both inhibitors should interfere with refilling of SR Ca^{2+} stores. Finally, mAChR-mediated Ca^{2+} waves always precede Ca^{2+} flashes/APs. Therefore, an increase in excitability, which occurs independently of IP_3-mediated Ca^{2+} signaling and results in Ca^{2+} influx during APs, mediates contraction induced by nerve-released ACh.

Neurally released ACh could increase excitability through Vm depolarization. Indeed, we observed an atropine-sensitive depolarization of ~2.4 mV following EFS in the presence of diltiazem that may be sufficient to trigger APs (18). The delayed onset of this depolarization seems independent of previous changes of membrane excitability, because, as we have shown previously (20), the muscarinic response has a slow onset in the presence as well as in the absence of P2X,R-induced changes in excitability. Although several studies have shown that activation of mAChRs causes an increase in excitability (5, 14), the mechanisms by which this occurs remain incompletely defined. One mechanism that has received considerable attention is the RhoA/Rho kinase pathway. The small GTPase RhoA can be activated by Cα_{q}-coupled receptors, such as M3 mAChRs (1), and studies have shown that the Rho kinase inhibitor Y27632 reduces carbachol-induced contractions in rat UBSM (22% reduction with 3 μM Y27632) (10). Similar inhibitory effects of Y27632 have been observed in mouse UBSM (43). However, Ding et al. (6) did not detect phosphorylation of the Rho kinase target (myosin phosphatase targeting subunit 1) in response to nerve stimulation of UBSM strips. Therefore, the mechanism by which ACh increases UBSM excitability remains elusive, including the identity of the ion channels that are the presumed targets of this pathway. In addition, other pathways, such as phospholipase A2 and phospholipase D (37), may also contribute.

**Conclusions.** In the present study, we present strong evidence that stimulation of mAChRs by nerve-released ACh causes rapid generation of IP_3,R-dependent Ca^{2+} waves during stimulation, and a profound increase in VDCC-dependent Ca^{2+} flashes/APs that have a much slower onset. Ca^{2+} waves cease after the stimulus ends, possibly reflecting rapid breakdown of IP_3 and ACh or activation of intracellular mechanisms.
that terminate G protein signaling (e.g., RGS proteins). We also show that nerve-released ACh increases UBSM excitability that does not depend on IP3-mediated Ca\(^{2+}\) release, and may be caused by membrane depolarization. Although the mechanisms linking mACHR activation with increased excitability in UBSM are not known, our data suggest that IP3, DAG, and depletion of PIP\(_2\) are not involved. In addition and contrary to conventional wisdom, our data do not support a role for IP3-mediated Ca\(^{2+}\) release in promoting contractility. Instead, our data strongly support increased excitability and subsequent APs in response to mACHR activation as the primary mechanism underlying contraction induced by stimulation of parasympathetic nerves.

**Perspective and Significance**

A fundamental function of the urinary bladder is to void urine. This occurs through activation of parasympathetic nerves to release ACh (and ATP) to contract UBSM. It has been accepted that nerve-released ACh acts on M3 mAChRs to cause contraction through IP3-mediated SR Ca\(^{2+}\) release. However, the linkage between this Ca\(^{2+}\) signal and UBSM contraction has been through association. Here, we challenge this dogma by examining the effects of neurally released ACh on Ca\(^{2+}\) signals and excitability and contractility of UBSM. Our data exclude a significant role for IP3-mediated Ca\(^{2+}\) signals in nerve-evoked contraction, and support the concept that ACh contracts UBSM through an increase in excitability (AP), which depends onVDCCs. Therefore, our data have important implications for urinary bladder function, and indicate new avenues of research to understand UBSM excitation-contraction coupling in health and disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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