Novel Excitatory Effects of Adenosine Triphosphate on Contractile and Pacemaker Activity in Rabbit Urethral Smooth Muscle

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Purpose: Adenosine triphosphate is thought to be an important neurotransmitter in urethral smooth muscle but its physiological role is still unclear. We characterized the effects of adenosine triphosphate on contractile and pacemaker activity in rabbit urethral smooth muscle.

Materials and Methods: Tension recordings were made from strips of rabbit proximal urethral smooth muscle. Membrane currents from freshly isolated smooth muscle cells and interstitial cells of Cajal were recorded using the patch clamp technique. Intracellular Ca^{2+} was measured using confocal microscopy.

Results: Exogenous application of adenosine triphosphate (10 μM) evoked robust contractions that were inhibited by the type 2 purinergic receptor blocker suramin (100 μM) and the selective type 2 purinergic Y1 receptor antagonist MRS2500 (Tocris Bioscience, Ellisville, Missouri) (100 nM). Application of the type 2 purinergic Y receptor agonist 2-MeSADP (1 μM) mimicked the effects of adenosine triphosphate. When smooth muscle cells were studied under voltage clamp at −60 mV, adenosine triphosphate evoked a large single inward current (greater than 1.2 nA) but 2-MeSADP produced a small current (about 16 pA). In contrast, when interstitial cells of Cajal were held at −60 mV, they showed spontaneous transient inward currents that were increased in frequency by adenosine triphosphate and 2-MeSADP. These excitatory effects were inhibited by suramin and MRS2500. Interstitial cells of Cajal showed spontaneous Ca^{2+} waves that were increased in frequency by adenosine triphosphate and 2-MeSADP. These effects were also inhibited by suramin and MRS2500.

Conclusions: Contractile effects of adenosine triphosphate in urethral smooth muscle are mediated by the activation of type 2 purinergic Y receptors on interstitial cells of Cajal.

Key Words: urethra; muscle, smooth; urinary incontinence; muscle contraction; adenosine triphosphate

Abbreviations and Acronyms

2-MeSADP = 2 methylthio adenosine diphosphate
ATP = adenosine triphosphate
AVP = arginine vasopressin
EFS = electrical field stimulation
ICC = interstitial cells of Cajal
NO-ARG = N^G-nitro-L-arginine
P2 = type 2 purinergic
SMC = smooth muscle cell
STIC = spontaneous transient inward current

Submitted for publication April 29, 2009.

Study received institutional animal care and use committee approval.

Supported by National Institutes of Health RO1 DK68565, Health Research Board PD/2005/4 and RP/2006/127, and the Council of Directors.

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URINARY continence depends on the ability of the urethra to generate closure pressure that exceeds intravesical pressure in the bladder. A key factor in this process is the ability of urethral smooth muscle to generate spontaneous tone.[1] The level of tone is regulated by several neurotransmitters, including noradrenaline, acetylcholine and nitric oxide.[2] ATP is also considered an important neurotransmitter in the urethra since studies show that it relaxes urethral smooth muscle strips.[3–8] However, since many of these studies were done in preparations precontracted with agonists, the

0022-5347/10/1832-0801/0
THE JOURNAL OF UROLOGY®

Vol. 183, 801-811, February 2010
Printed in U.S.A.
DOI:10.1016/j.juro.2009.09.075

effects of ATP on urethral tone under resting conditions have not yet been fully characterized and its physiological role in the urethra remains unclear. Callahan and Creed noted that the inhibitory effects of ATP on urethral smooth muscle were only observed in preparations in which tone had been raised by prior application of noradrenaline or noradrenaline and quinidine.[3] When ATP was applied to muscle strips under resting conditions, it produced a small contraction but this response was not investigated further. Thus, we investigated the effects of ATP on urethral contractility at resting tone.

Spontaneous myogenic tone in the urethra is associated with spontaneous transient depolarizations and large, regularly occurring slow waves.[9,10] Sergeant et al suggested that this activity originates in specialized pacemaker cells termed ICC.[11] The frequency of pacemaker activity in ICC is increased by noradrenaline[11,12] and decreased by nitric oxide,[13] leading to the suggestion that ICC may also act as targets for neurotransmitters in the urethra, as in the gastrointestinal tract.[14] Thus, we characterized the effects of ATP on urethral muscle strips under resting conditions and investigated whether spontaneous activity in isolated urethral ICC is modulated by ATP.

MATERIALS AND METHODS

Cell Isolation

Male and female New Zealand White rabbits at ages 16 to 20 weeks were humanely sacrificed with a lethal intravenous injection of pentobarbitone. The most proximal 1.5 cm of the urethra were removed and placed in Krebs solution. Individual ICC and SMC were isolated enzymatically, as described previously.[11] All procedures were done with the approval of the institutional animal care and use committee.

Tension Recordings

Strips of circular and longitudinally oriented smooth muscle 8 × 1 × 1 mm were removed from the urethra, placed in water jacketed organ baths maintained at 37C and perfused with Krebs solution bubbled with 95% O_2-5% CO_2 containing atropine (1 μM), phentolamine (1 μM) and NO-ARG (Calbiochem®) (100 μM). Strips were adjusted to a tension of 2 to 4 mN and allowed to equilibrate for 50 minutes before experiments. Contractions were measured using the multi channel Myobath system (WPI-Europe, Stevenage, United Kingdom). Data were acquired using Data-Trax™ 2. Transmural nerve stimulation was applied by a D330 MultiStim system stimulator (Digitimer, Welwyn Garden City, United Kingdom), which delivered 0.3-millesecond pulses of 20 V (nominal) at a frequency of 4 Hz for 30 seconds.

Patch Clamp Recordings

Currents were recorded using the perforated patch configuration of the whole cell patch clamp technique.[15] The membrane was perforated using the antibiotic amphotericin B (600 μg/ml). Pipettes were pulled from borosilicate glass capillary tubing (Clark Electromedical Instruments, Edenbridge, United Kingdom) (inner and outer diameter 1.17 and 1.5 mm, respectively) to a tip of diameter approximately 1 to 1.5 μm with a resistance of 2 to 4 MΩ. Voltage clamp commands were delivered by an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, California) connected to a Digidata® 1322A AD/DA converter interfaced to a computer running pClamp™. During experiments the dish containing cells was superfused with Hanks solution. The cell under study was also continuously superfused with Hanks solution by a close delivery system consisting of a pipette (tip diameter 200 μm) approximately 300 μm away. With a dead space time of around 5 seconds this could be changed to a solution containing a drug. All experiments were done at 35C to 37C.

Ca Imaging

Isolated cells were incubated in 0.4 μM fluo-4/AM (Molecular Probes®) in Hanks solution containing 100 μM Ca^{2+}

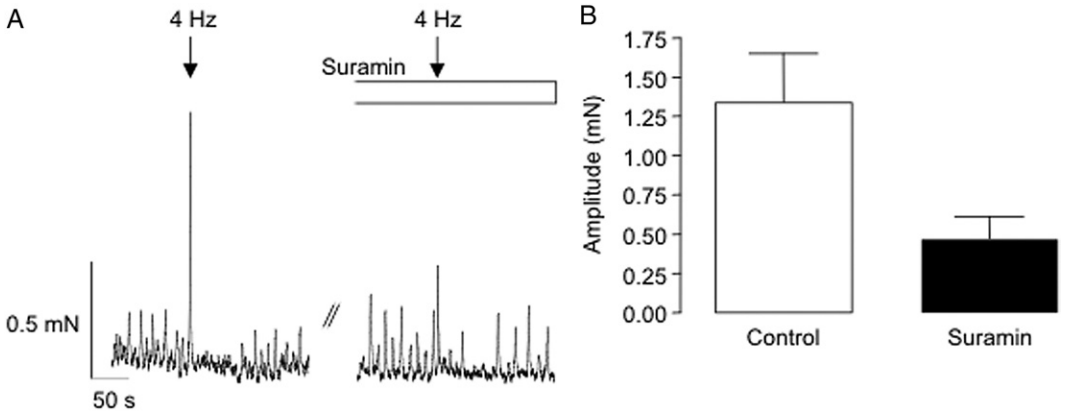

Figure 1. Effect of suramin on EFS induced contractions of rabbit urethral smooth muscle. *A,* 0.3-millisecond pulse duration and 4 Hz frequency for 30 seconds (*s*) produced transient contraction that was attenuated by 100 μM suramin. *B,* contraction amplitude plotted in absence (*Control*) and presence of suramin in 11 experiments.

for 6 to 8 minutes in the dark at room temperature. Cells were imaged using an iXon 887 EMCCD camera (Andor Technology, Belfast, United Kingdom) (512 × 512 pixels with a pixel size of 16 × 16 μm) coupled to a CSU22 Nipkow spinning disk confocal head (Yokogawa, Tokyo, Japan). A krypton-argon laser (Melles Griot, Wellingborough, United Kingdom) at 488 nm was used to excite fluo-4 and emitted light was detected at wavelengths greater than 510 nm. Experiments were done with a 60× objective (Olympus®), resulting in images with a pixel resolution of 0.266 × 0.266 μm. Images were acquired at 5 fps. Background fluorescence from the camera, obtained

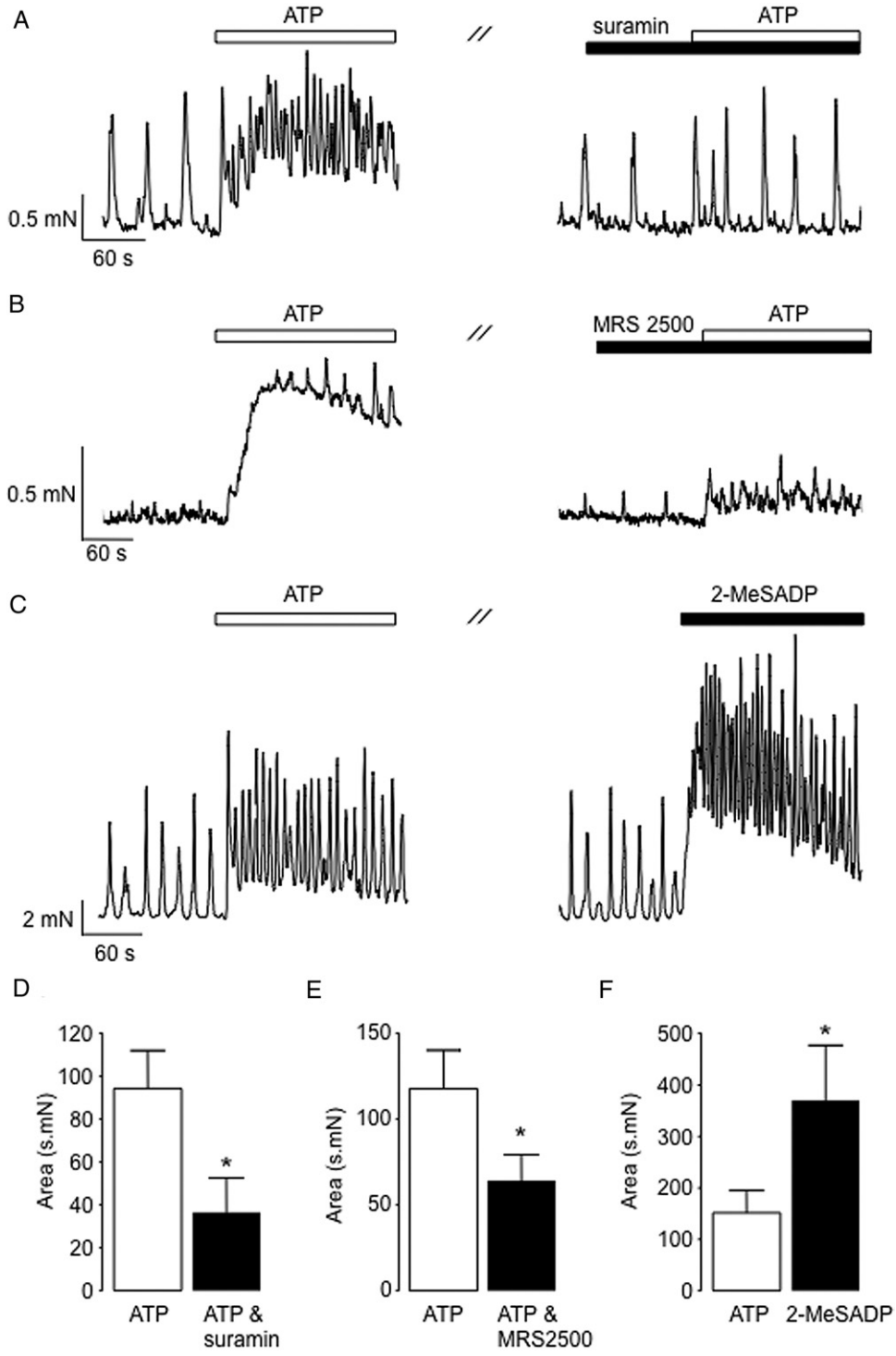

Figure 2. Effect of ATP on contractile activity in rabbit urethral smooth muscle strips. *A* and *B*, representative traces show that suramin and MRS2500 inhibited ATP induced contractions. *s*, seconds. *C*, representative record shows that 2-MeSADP mimicked ATP contractile effects. *D* to *F*, summary data.

using a null frame, was subtracted to determine fluorescence (F) with F_0 considered the minimum fluorescence measured between oscillations under control conditions. Post hoc line scan images were obtained from a 1 pixel thick line drawn centrally through the entire cell length and using the reslice command in ImageJ. A spatial calibration bar representing 40 μm was shown in yellow at the right hand side of each image. Plots of F/F_0 were obtained from the post hoc line scan by drawing a 1 pixel thick line across the line scan image at the point indicated by the arrow at the left side of each image and plotting the intensity profile in ImageJ.

Solutions and Drugs

Hanks solution was composed of 130 mM Na^+, 5.8 mM K^+, 135 mM Cl^-, 4.16 mM HCO_3^-, 0.34 mM HPO_3^{2-}, 0.44 mM H_2PO^{4-}, 1.8 mM Ca^{2+}, 0.9 mM Mg^{2+}, 0.4 mM SO_4^{2-}, 10 mM dextrose, 2.9 mM sucrose and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Ca^{2+}-free Hanks solution for cell dispersal was composed of 125 mM NaCl, 5.36 mM KCl, 10 mM glucose, 2.9 mM sucrose, 15.5 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4 and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Krebs solution was composed of 120 mM NaCl, 5.9 mM KCl, 1.2 mM $NaHCO_3$, 5.5 mM glucose, 2.5 mM $CaCl_2$ and 1.2 mM $MgCl_2$, pH maintained at 7.4 by bubbling with 95% O_2-5% CO_2. Perforated patch solution was composed of 133 mM CsCl, 1.0 mM $MgCl_2$, 0.5 mM ethylene glycol tetraacetic acid and 10 mM HEPES, pH adjusted to 7.2 with CsOH. Drugs used were ATP, suramin, phentolamine, atropine, tetrodotoxin (Sigma®), 2-MeSADP, AVP, NO-ARG and MRS2500.

Statistics

Summary data are shown as the mean ± SEM. Statistical differences in all experiments were compared using Student's paired t test with $p < 0.05$ considered significant. In each experimental series the number of cells was obtained from a minimum of 2 animals.

RESULTS

Tension Recordings

Figure 1 shows that EFS of intramural nerves (in the presence of atropine, phentolamine and NO-ARG to block contributions from cholinergic, adrenergic and nitrergic nerves) produced transient urethral smooth muscle contractions that were significantly attenuated in 11 muscle strips by the broad-spectrum P2 receptor antagonist suramin (100 μM) ($p < 0.05$). EFS induced contractions in 8 muscle strips were also inhibited by tetrodotoxin (1 μM) from a mean of 1.1 ± 0.3 to 0.03 ± 0.02 mN (data not shown, $p < 0.05$), suggesting that ATP or a related purine

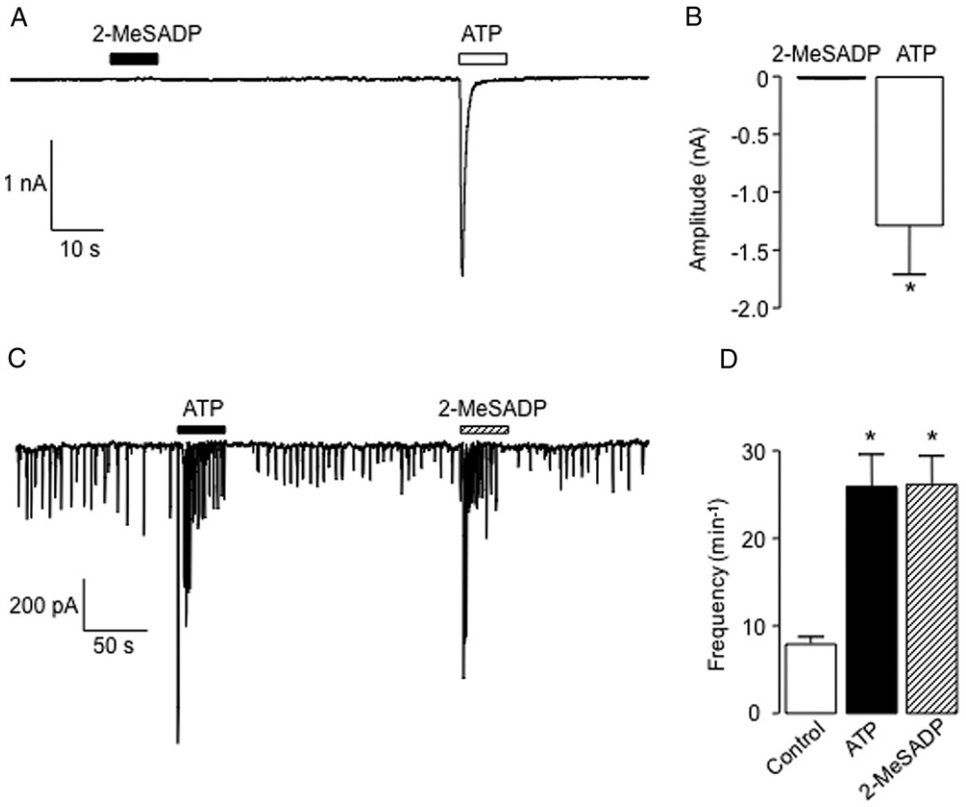

Figure 3. Effect of ATP and 2-MeSADP on inward currents recorded in isolated urethral SMCs and ICC. *A*, representative trace shows that ATP but not 2-MeSADP evoked large transient inward current in urethral SMCs voltage clamped at −60 mV. *s*, seconds. *B*, mean amplitude of currents evoked by ATP and 2-MeSADP. *C*, representative trace shows that ATP and 2-MeSADP increased STIC frequency in ICC under voltage clamp at −60 mV. *D*, summarized data.

may act as an excitatory neurotransmitter in urethral smooth muscle. This idea was supported by the finding that exogenous application of ATP (10 μM) induced robust contractions (fig. 2). These responses typically consisted of an increase in tone with superimposed transient phasic contractions that varied in frequency and amplitude. These effects were quantified by measuring total area under the trace in mN per second for 3 minutes immediately before and during ATP application. ATP significantly increased mean contractile activity in 45 muscle strips from 43.6 ± 20.8 to 163 ± 40.1 mN per second ($p < 0.05$). The representative trace and summary data revealed that the contractile effects of exogenous ATP application were also inhibited by suramin (100 μM) (fig. 2, *A* and *D*). In 17 preparations suramin significantly decreased the mean ATP induced contraction from 95 ± 18 to 37 ± 16 mN per second ($p < 0.05$).

P2 receptors are classified into the 2 broad categories P2X and P2Y.[16,17] Several P2Y receptor isoforms are expressed in urethral smooth muscle but their role is unclear.[7,18] To test whether they contributed to the ATP induced contraction we examined the effects of the P2Y1 receptor antagonist MRS2500.[19] MRS2500 (100 nM) inhibited the ATP induced contraction (fig. 2, *B* and *E*). In 18 muscle strips the mean ATP evoked contraction was decreased from 118 ± 23 to 65 ± 15 mN per second by MRS2500 ($p < 0.05$). The role of P2Y receptors was further tested using the P2Y receptor agonist 2-MeSADP.[20] 2-MeSADP (1 μM) induced a contraction similar to that evoked by ATP (10 μM) (fig. 2, *C*). This effect was representative of 14 muscle strips in which the mean contraction amplitude induced by 2-MeSADP was 372 ± 108 vs the 153 ± 44 mN per second induced by ATP ($p < 0.05$). Together these data suggest that the excitatory effects of ATP are mediated by P2Y receptors.

Voltage Clamp Experiments

The cellular basis of ATP and 2-MeSADP induced contractions was examined by investigating their effects on inward currents in freshly isolated SMCs and ICC. ATP evoked a large, single inward current in SMCs held at −60 mV but 2-MeSADP did not produce a resolvable response (fig. 3, *A*). The mean amplitude of the ATP current measured in 7 SMCs was −1,291 ± 422 vs −16 ± 8 pA in response to 2-MeSADP (fig. 3, *B*). In contrast to SMCs, ICC were spontaneously active and equally sensitive to ATP

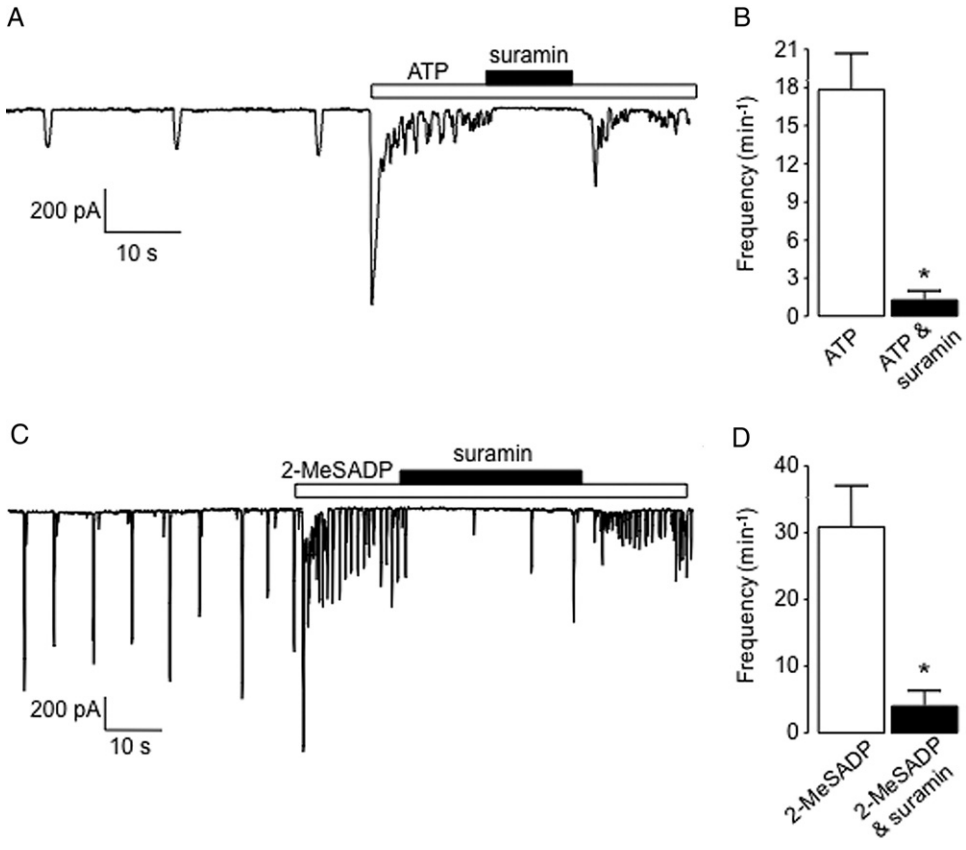

Figure 4. Effect of suramin on STICs induced by ATP and 2-MeSADP in ICC. *A,* representative trace shows that suramin inhibited STICs induced by ATP. *s,* seconds. *B,* mean STIC frequency induced by ATP in absence and presence of suramin. *C,* representative trace shows that suramin inhibited STICs induced by 2-MeSADP. *D,* mean STIC frequency induced by 2-MeSADP in absence and presence of suramin.

and 2-MeSADP. Each drug induced a series of oscillatory inward currents that were initially larger in amplitude than control activity but decreased during the exposure period (fig. 3, *C*). These currents occurred at a much greater frequency than control activity. In 6 cells ATP significantly increased the frequency from 9 ± 1.6 to 26 ± 3.7 minutes^{-1} ($p < 0.05$). In 8 cells 2-MeSADP caused a significant increase from 7.25 ± 0.5 to 26.25 ± 3.2 minutes^{-1} ($p < 0.05$, fig. 3, *D*).

We then investigated the sensitivity of ATP responses in ICC to suramin and MRS2500. Suramin application in the continued presence of ATP or 2-MeSADP reversibly inhibited the evoked currents (fig. 4, *A* and *C*). Summary data from 9 cells showed that suramin decreased the mean frequency of ATP evoked currents from 17.9 ± 2.8 to 1.3 ± 0.66 minutes^{-1} and decreased those evoked by 2-MeSADP in 6 cells from 31 ± 6.1 to 4.2 ± 2.1 minute^{-1} (each $p < 0.05$, fig. 4, *B* and *D*). MRS2500 (100 nM) also inhibited the excitatory effects of ATP and 2-MeSADP. Figure 5, *A* and *C* shows representative traces of this effect. Figure 5, *B* and *D* show corresponding summary data. MRS2500 in 8 cells decreased the mean frequency of ATP evoked inward currents from 17 ± 4 to 6 ± 2.5 minutes^{-1} and decreased those evoked by 2-MeSADP in 6 cells from 25.8 ± 4.3 to 7 ± 1.8 minutes^{-1} (each $p < 0.05$).

In contrast to the effects of suramin and MRS2500 on ATP and 2-MeSADP evoked currents, they did not significantly affect spontaneous activity in ICC (fig. 6, *A* and *C*). In 8 cells tested the frequency of STICs before adding suramin was 12.1 ± 2.5 vs 9.4 ± 1.9 minutes^{-1} in its presence ($p > 0.05$). The mean frequency of STICs in 7 cells in the absence of MRS2500 was 8.4 ± 2.5 vs 9.1 ± 3.13 minutes^{-1} in its presence ($p > 0.05$, fig. 6, *B* and *D*).

Ca^{2+} Imaging Experiments

Electrical activity in urethral ICC is initiated by spontaneous Ca^{2+} oscillations.[12,21] Thus, we then investigated whether this activity was also modulated by ATP and 2-MeSADP. Post hoc line scan images and associated intensity profile plots showed that ATP and 2-MeSADP dramatically increased the frequency of these events (fig. 7, *A* and *B*). ATP significantly increased the mean frequency of Ca^{2+} waves from 9 ± 1.4 to 29.1 ± 4.5 minutes^{-1} in 14 cells and 2-MeSADP resulted in an increase from 7.3 ± 1.6 to 17.1 ± 2.8 minutes^{-1} in 10 cells

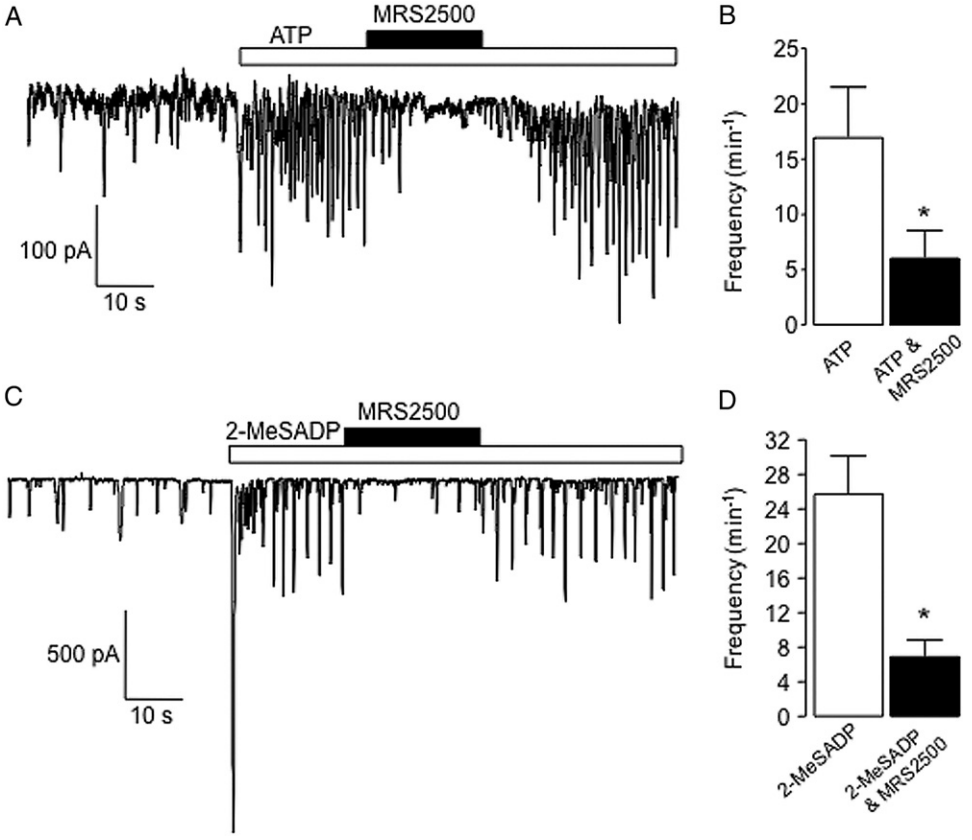

Figure 5. Effect of MRS2500 on STICs induced by ATP and 2-MeSADP in ICC. *A,* representative trace shows that MRS2500 inhibited STICs induced by ATP. *s,* seconds. *B,* mean STIC frequency induced by ATP in absence and presence of MRS2500. *C,* representative trace shows that MRS2500 inhibited STICs induced by 2-MeSADP. *D,* mean STIC frequency induced by 2-MeSADP in absence and presence of MRS2500.

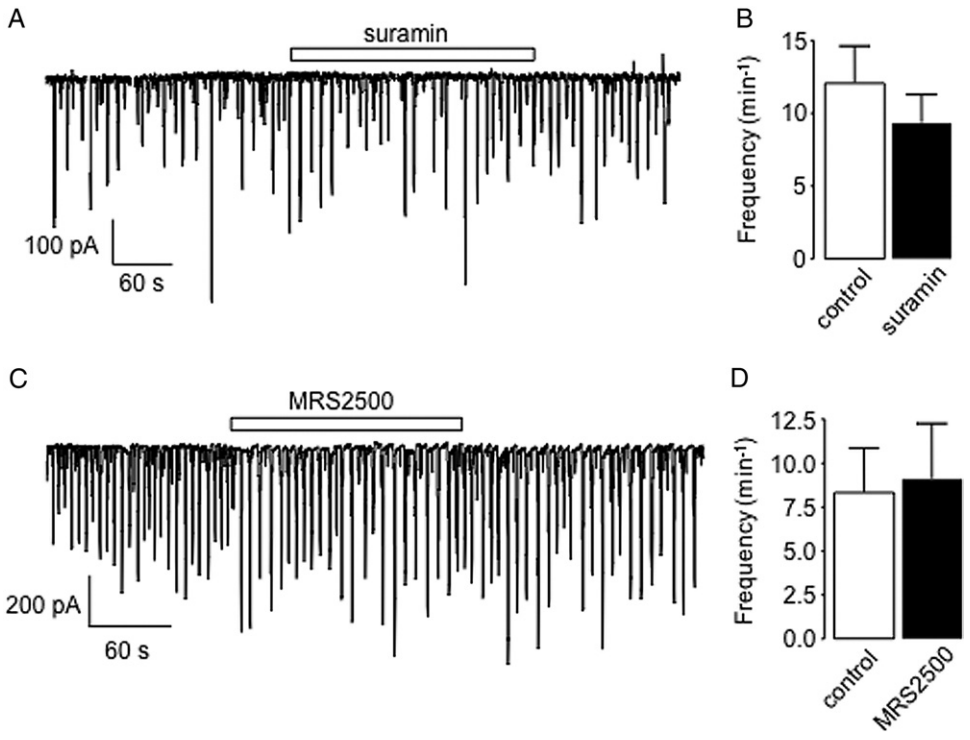

Figure 6. Effect of suramin and MRS2500 on STICs in isolated urethral ICC. *A* and *C,* representative traces show effect at −60 mV. *s,* seconds. *B* and *D,* mean STIC frequency in absence and presence of suramin and MRS2500.

($p < 0.05$, fig. 7, *C* and *D*). ATP evoked Ca^{2+} oscillations were inhibited by suramin and MRS2500 (figs. 8, *A* and 9, *A*). Suramin decreased the mean frequency of ATP induced Ca^{2+} waves from 27 ± 8.7 to 6.6 ± 2.1 minutes^{-1} in 7 preparations and MRS2500 caused a decrease from 28.1 ± 4.2 to 10.3 ± 3.9 minutes^{-1} in 6 (each $p < 0.05$, figs. 8, *B* and 9, *B*).

In contrast to these results, previous studies show that ATP produced relaxant effects in urethral smooth muscle.[3–8] Most of these studies were performed in muscle strips precontracted with agents such as AVP or the prostaglandin peroxidase inhibitor U46619. To test whether precontraction could account for these differences we compared the effect of ATP before and during contraction with AVP. ATP caused a contraction when applied before AVP but a transient contraction followed by longer relaxation when contracted with AVP (fig. 10).

DISCUSSION

ATP is widely regarded to have an important part in the neural regulation of urine storage and voiding. However, while its effects on bladder function are well characterized,[22–24] its role in the urethra is less clear. We report that ATP increased the frequency of pacemaker activity in isolated urethral ICC and enhanced tonic and phasic contractile activity in rabbit urethral smooth muscle strips. These effects were mimicked by the P2Y receptor agonist 2-MeSADP and inhibited by the broad-spectrum purinergic receptor inhibitor suramin and the selective P2Y1 receptor antagonist MRS2500, suggesting that they were caused by P2Y receptor activation.

ATP induces detrusor smooth muscle contraction via activation of a P2X mediated inward current in SMCs.[23,25,26] However, while we observed a similar current in isolated urethral SMCs, it seems unlikely to account for the ATP induced contraction since it was not mimicked by 2-MeSADP, which potently contracted strips of urethral smooth muscle. An alternative possibility is that ATP induced urethral smooth muscle contraction could be mediated by effects in ICC.

Urethral ICC are putative pacemaker cells that develop spontaneous electrical depolarizations that are then conducted to the bulk smooth muscle.[11] Studies show that the frequency of this activity is modulated by several neurotransmitters. For example, noradrenaline, the principal excitatory neurotransmitter in urethral smooth muscle, increases the frequency of spontaneous transient depolarizations and STICs in freshly dispersed ICC while NO, the main inhibitory neurotransmitter, decreases the frequency of activity.[11–13] These observations led to the hypothesis that ICC in the urethra could act as targets for autonomic neurotransmitters, which

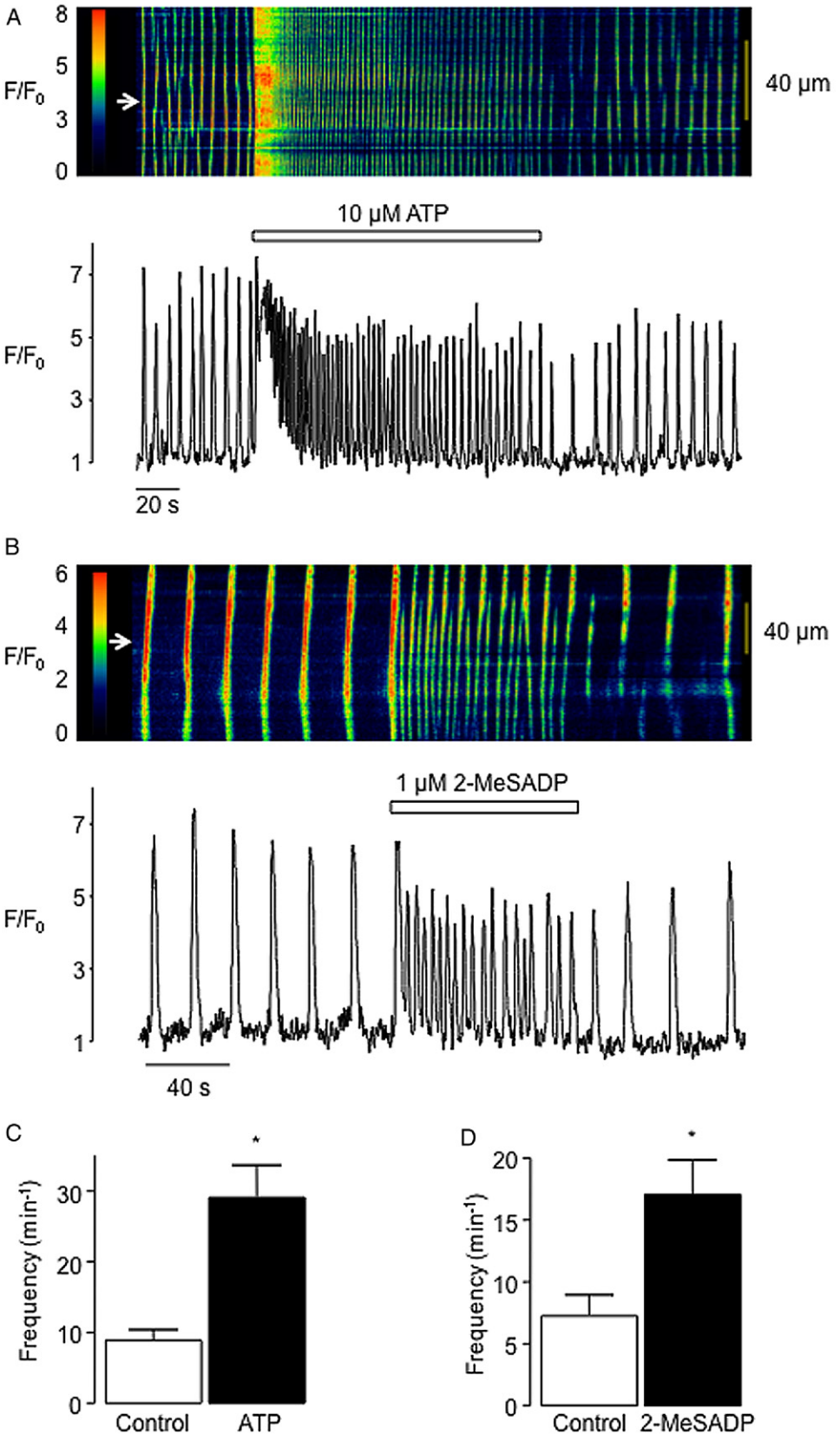

Figure 7. Effect of ATP and 2-MeSADP on spontaneous Ca^{2+} oscillations in isolated urethral ICC. *A* and *B*, post hoc line scan and intensity profile plots. *s*, seconds. *C* and *D*, summary plots on wave frequency.

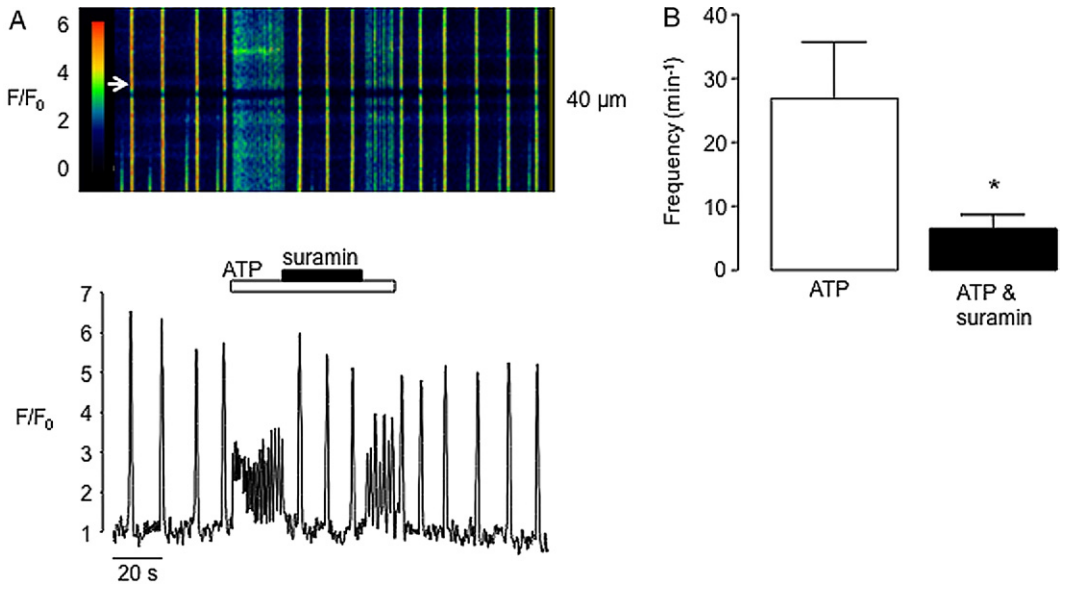

Figure 8. Effect of suramin on ATP induced Ca^{2+} oscillations in isolated urethral ICC. *A,* post hoc line scan and intensity profile plot show effect of 100 μM suramin. *s,* seconds. *B,* mean wave frequency induced by ATP in absence and presence of suramin.

then regulate myogenic tone by affecting the frequency of pacemaker activity. Our results are consistent with this idea. Thus, exogenous application of ATP increased the frequency of STICs and Ca^{2+} waves in isolated ICC, and the pharmacological profile of this response matched that of the contractile response. To our knowledge it is currently unknown whether ICC in the urethra are innervated, although several studies show that they form close associations with nerves, indicating that they could be involved with neurotransmission.[27,28] Also, since ICC are known to mediate neural responses in the gastrointestinal tract,[14] it is possible that urethral ICC could perform a similar role.

The contractile effects of ATP in our study are in contrast to those in previous reports showing that ATP induced urethral smooth muscle relaxation.[5–8] However, in an earlier study Callahan and Creed suggested that the effect of ATP on urethral contractility depends on the degree of muscle tone.[3] Our

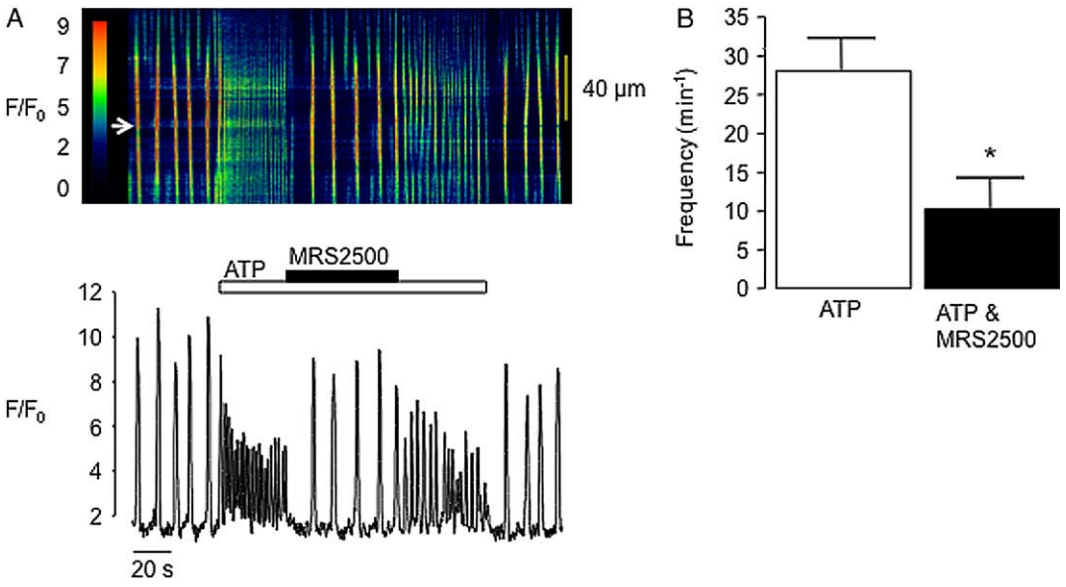

Figure 9. Effect of MRS2500 on ATP induced Ca^{2+} oscillations in isolated urethra ICC. *A,* post hoc line scan and intensity profile plot show effect of 100 nM MRS2500. *s,* seconds. *B,* mean wave frequency induced by ATP in absence and presence of MRS2500.

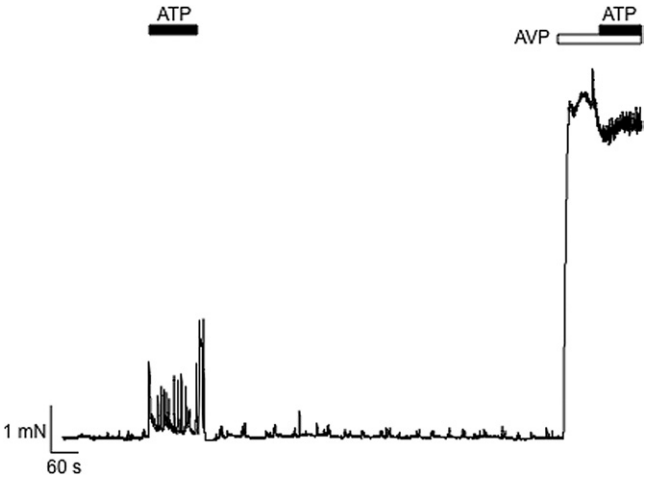

Figure 10. Representative recording shows effect of ATP on urethral contractility when applied before and during precontraction with AVP. *s*, seconds.

results are in agreement with their suggestion. For example, while ATP contracted muscle strips when applied at resting tone, it relaxed the tissue after tone was raised by prior application of AVP. These effects are reminiscent of observations made in the pulmonary circulation, in which P2Y receptor agonist application induced vasoconstriction at resting tone and vasodilation when muscle tone was increased with phenylephrine.[29,30] Thus, the net effect of ATP on urethral smooth muscle may be influenced by the degree of muscle tone. However, some investigators reported ATP induced relaxation of urethral smooth muscle even in the absence of precontraction.[4] Therefore, the differential effects of ATP on urethral contractility may also depend on other factors.

CONCLUSIONS

Our results reveal that ATP can induce urethral smooth muscle contraction, most likely via the activation of P2Y receptors. Also, ATP can increase the frequency of spontaneous Ca^{2+} oscillations and inward currents in isolated ICC, and the pharmacological profile of these responses matched that of the contractile response. These data indicate that ATP is an excitatory neurotransmitter in rabbit urethral smooth muscle that may mediate its effects by regulating the frequency of pacemaker activity in ICC.

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