The purinergic component of human vas deferens contraction

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Objective: To examine purinergic signaling in human vas deferens.
Design: To study contractile responses of the scrotal vas deferens.
Setting: Research department of a university teaching hospital.
Patient(s): Undergoing vasectomy or orchidectomy (aged 27–88 years, n = 14).
Intervention(s): Vasectomy or orchidectomy.
Main Outcome Measure(s): Strips of vas deferens were suspended in an organ bath and subjected to electrical stimulation to establish frequency–response curves. These stimulations were repeated in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, P2 receptor antagonist), prazosin (adrenergic α_1 antagonist), and tetrodotoxin. Concentration–response curves were constructed to noradrenaline and the P2X agonists ATP and α,β-methylene ATP (α,β-meATP). The P2X receptor subtype distribution was assessed by immunohistochemistry using specific antibodies.
Result(s): The response at 32 Hz in the presence of PPADS was reduced by 40% and in the presence of prazosin by 80%. Noradrenaline caused concentration-dependent contractions (EC_{50} = 11.8 μM). Contractions to ATP and α,β-meATP (EC_{50} = 6.27 μM) suggested that the functional receptor was $P2X_1$ and/or $P2X_3$. However, immunohistochemistry demonstrated $P2X_1$, but not $P2X_3$, receptor immunoreactivity on the smooth muscle cells.
Conclusion(s): This study demonstrated that ATP is a co-transmitter with noradrenaline in the contraction of the human vas deferens predominantly acting through the $P2X_1$ receptor. (Fertil Steril® 2006;85:932–9. ©2006 by American Society for Reproductive Medicine.)

Key Words: Human, vas deferens, ATP, noradrenaline, P2X, co-transmission, smooth muscle contraction

Adenosine 5′-triphosphate (ATP), co-released with noradrenaline (NA) from sympathetic nerves and acetylcholine (ACh) from parasympathetic nerves, acting through P2X receptors, has been shown to cause contraction of genitourinary smooth muscle of mammals (1, 2). This purinergic component of smooth muscle contraction is variable between tissues and species. The functional importance of co-released ATP that has been demonstrated in laboratory animals is not always mirrored in human tissue. For instance, in the rabbit bladder the purinergic component is accountable for up to 40% of the smooth muscle contraction (3), and in the guinea pig vas deferens up to 70% (4). In the normal human bladder it is very small, although in the pathological states such as detrusor instability and interstitial cystitis, the purinergic component becomes significant, accounting for up to 40% of the contraction (5–7).

ATP acts through P2 receptors; these have been divided into P2X and P2Y families (8–10). P2X receptors are ionotropic whereas P2Y receptors are G protein-coupled. Seven subtypes of the P2X family have been cloned and characterised ($P2X_{1-7}$) and eight subtypes of the P2Y family have been identified (11–13).

Noradrenaline and ATP have been shown to mediate contraction of the rat and guinea pig vas deferens smooth muscle. The contraction, in response to electrical field stimulation (EFS) of sympathetic nerves has been shown to be biphasic (4, 14, 15). ATP, released from sympathetic nerves, acts through ion-gated P2X receptors to stimulate the initial fast phase of the contraction, with co-released NA causing the more sustained, but slower contraction, acting through G protein-coupled α-adrenoceptors.

The $P2X_1$ receptors have been demonstrated on the membranes of smooth muscles in many organs including the vas deferens (16–20), where it mediates contraction. This has

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been supported by the demonstration that $P2X_1$ receptor-null mice were shown to have a 90% reduction in fertility due to reduced nerve-induced contractions of the smooth muscle, although their sperm were fertile (20). The smooth muscle was still contractile to NA, but resulting ejaculates had insufficient sperm for fertilization. It was subsequently suggested that $P2X_1$ antagonists could be developed for male contraception.

Contraction of the human vas deferens smooth muscle has been reported to be under purely adrenergic control, although modified by numerous substances (21, 22) and about 10 times slower than those of the rat. Twitch responses to single shocks are tetrodotoxin (TTX)-insensitive, but electrically induced tetanic responses have been shown to be sensitive to prazosin and TTX, although up to 23% of the original response persisted. Some sections of the human vas deferens were shown to relax in response to EFS, although this occurred at low voltages, and was atropine sensitive (23, 24). Exogenous NA causes tonic contractions, induces spontaneous contractions, and potentiates the response to electrical stimulation (25).

The role of ATP as a co-transmitter and the distribution of the $P2X_1$ receptor in the human vas deferens have yet to be fully examined. This study examined the purinergic component of human vas deferens smooth muscle contraction in response to EFS of autonomic nerves, and exogenous agonists. The tissue distribution of P2X receptor subtypes was also examined by immunohistochemistry.

MATERIALS AND METHODS

Pharmacology

Institutional review board approval was not obtained as this study was undertaken outside of the United States. Equivalent standards were maintained, and hospital ethical approval was obtained for the study, all patients were fully informed, and individual consent for tissue experimentation was gained before their operation.

Approximately 2-cm sections of scrotal vas deferens were collected in Krebs solution (composition (in millimoles per liter): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.4; $MgSO_4$, 0.6; NaH_2PO_4, 1.4; glucose, 7.7 and $CaCl_2$, 2.5, at pH 7.3) from patients undergoing either vasectomy or orchidectomy (age range, 27–88 years; n = 14). All experiments were started within 2 hours of tissue collection. Each section of vas deferens was divided into quarters longitudinally and attached to a force displacement transducer (FT03C, Grass Instruments, Quincy, MA) in a Krebs solution-filled 10-mL organ bath, continually gased (95% O_2/5% CO_2). Nerve stimulation of the strips was facilitated by two platinum wire rings 2.5 mm in diameter and 1 cm apart through which the preparations were threaded. Isometric contractions were recorded using the software PowerLab Chart for Windows (version 4; AD Instruments, Bella Vista, New South Wales, Australia). Experiments were carried out at 36° ± 1°C to approximate scrotal temperature. An initial load of 1 g was applied to the strips, which were allowed to equilibrate for at least 1 hour before the start of the experiment. Separate experiments were carried out to evaluate contraction due to EFS of sympathetic nerves (n = 7, strips = 10), and exogenously applied agonists; NA (n = 14, strips = 35), ATP (n = 12, strips = 31), and the slowly hydrolyzable ATP analogue α,β-methylene ATP (α,β-meATP) (n = 11, strips = 27). The contraction due to a standard concentration of 120 mM potassium chloride (KCl) was noted at the end of each experiment.

Drugs Used

ATP, α,β-meATP (lithium salt), NA, prazosin, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and TTX were obtained from Sigma Chemical Co. (Poole, UK). Stock solutions were prepared in distilled water except for NA, which was prepared in ascorbic acid (1 μM). The volume added to the organ bath to produce the final concentration was not in excess of 100 μL.

Frequency–Response Curves

Strips of vas deferens were subjected to EFS (100 V, 0.5 milliseconds, 1–64 Hz, 20 seconds) every 5 minutes. Three frequency–response curves were carried out to establish consistent contractile responses. Frequency–response curves were subsequently constructed after 20 minutes in the presence of the α_1-adrenoceptor antagonist prazosin (1 μM) or the P2X receptor antagonist PPADS (30 μM), to establish the relative adrenergic and purinergic components of the contraction. Additional frequency–response curves were then constructed in the presence of both antagonists, and then finally in the presence of TTX (1 μM), to determine the part of the response due to direct electrical stimulation of smooth muscle. The Krebs solution in the organ bath was changed between each set of frequency–response curves.

Concentration–Response Curves

Noncumulative concentration–response curves were constructed for NA (0.1–300 μM), ATP (0.1 μM–1 mM), and α,β-meATP (0.1–300 μM). Boluses of α,β-meATP were applied at least 20 minutes apart to avoid desensitization of the $P2X_1$ receptor.

Statistical Analysis

Responses to EFS were corrected to take into account the TTX-insensitive component of the contractions. The contraction to EFS is expressed as mean percentage contraction of the maximum contraction ± SEM (n). Contractile responses to noncumulatively applied agonists were expressed as mean percentage of the maximum response to KCl (120 mM) ± SEM (n). Because the concentration–response curve to ATP did not reach a maximum, it was not possible to calculate an EC_{50} concentration.

Statistical analysis was carried out using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Statistical significance was tested by a two-way analysis of variance (ANOVA) followed by a post hoc test (Bonferroni). A probability of $P<.05$ was considered significant.

Immunohistochemistry

Tissue samples of vas deferens were embedded in Tissue Tek OCT compound (Sakura, Zoederwoude, Netherlands) and snap-frozen in isopentane precooled liquid nitrogen. The tissues were sectioned at 12 μm using a cryostat (Leica CM 3050, Nussloch, Switzerland), thaw mounted on gelatin-coated slides and air-dried at room temperature. The slides were stored at -20°C and allowed to return to room temperature for at least 10 minutes before use.

Antibodies

The immunogens used were peptides corresponding to 15 receptor type-specific amino acids in the carboxy-terminal region of the P2X receptor subtypes (Roche Bioscience, Palo Alto, CA). The specificity of the antibodies was verified by immunoblotting as previously described (26).

Technique

The avidin–biotin technique was used, as described by Llewellyn-Smith et al. (27, 28). Briefly, 12-μm sections were fixed in 4% formaldehyde and 0.2% of a saturated picric acid solution in 0.1 M phosphate buffer for 2 minutes. Endogenous peroxidase was inactivated by incubating with 50% methanol containing 0.4% hydrogen peroxide for 10 minutes. Nonspecific binding sites were blocked by incubating with 10% normal horse serum in phosphate buffered saline (PBS) containing 0.05% thimerosal (Merthiolate) for 20 minutes. The P2X antibodies were diluted to 5 μg/mL (determined by prior titration) with normal horse serum and the sections were incubated with primary antibodies overnight at room temperature. The secondary antibody was a biotinylated donkey antirabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) used at 1:500 for 1 hour. Sections underwent a further incubation with extravidin peroxidase (Sigma) at 1:1000 for 1 hour. The reaction product was visualized using the nickel-DAB enhancement technique. The specimens were dehydrated in xylene and mounted in Eukitt (BDH). Controls were performed with preimmune IgG and antibodies preadsorbed with the homologous peptides and omission of the primary antibody. The results were documented using the Edge R400 high definition light microscope (Edge Scientific Instruments, Santa Monica, CA). Images were taken with a Leica DC 200 digital camera (Leica 2000, Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) and imported into a graphics package (Adobe Photoshop 5.0, San Jose, CA).

RESULTS

Pharmacology

The EFS of sympathetic nerves of the vas deferens induced frequency-dependent contractions that were predominantly monophasic (Fig. 1a,d), but became rhythmic at the highest frequencies. Maximum contraction was achieved at 32 Hz. The neurogenic contraction was significantly reduced by prazosin by more than 80% at peak frequency (Fig. 1b, Fig. 2a) ($P=.0001$). The EFS-induced contractions in the presence of PPADS (30 μM) were also significantly reduced ($P<.0001$; Fig. 1e, Fig. 2a) from the initial contraction (Fig. 1d, Fig. 2a) being more evident at the higher frequencies; at 32 Hz this amounted to a 40% reduction from the initial contraction. Virtually all of the neurogenic contraction was abolished in the presence of both PPADS and prazosin (Fig. 1f). At the lower frequencies tested prazosin almost completely abolished the smooth muscle contraction and PPADS caused minimal antagonism. At the higher frequencies tested PPADS caused relatively greater antagonism and prazosin relatively less (Fig. 2a). In the presence of TTX, some residual contraction remined, but only at the highest frequencies tested (Fig. 1c), confirming that most of the contraction was nerve-mediated.

Noradrenaline produced a concentration-dependent contraction of the vas deferens. The contractile responses were typically slow rising, with a stimulus to maximal response time of approximately 12 seconds (Fig. 1g). Suprathreshold rhythmic contractions developed when concentrations at more than 10 μM were used. The EC_{50} value was 11.8 μM (Fig. 2b).

The α,β-meATP caused contractions similar to those induced by ATP, but were larger and more consistent (Fig. 1h). Concentration–response curves obtained for α,β-meATP did reach a maximum (Fig. 2c). Although some desensitization was noted at the highest concentrations used, the EC_{50} value was calculated to be 6.27 μM. ATP induced small, monophasic responses (Fig. 1i), which were less consistent and no maximum contraction was obtained despite using concentrations up to 1 mM (Fig. 2c).

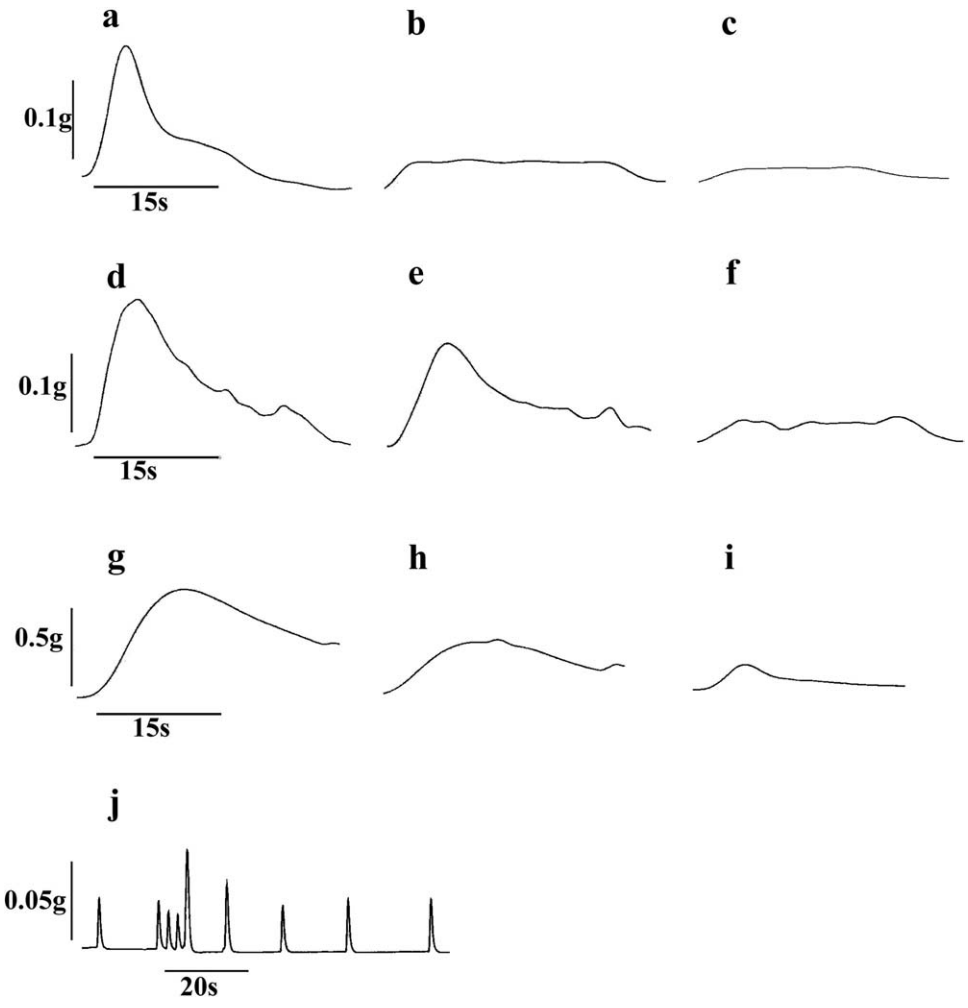
Spikes of spontaneous rhythmic contractile activity were observed in 9/14 vas deferens preparations, averaging 100 contractions per hour, with a mean contractile force of 5.8 mg/mg tissue. ATP, at subthreshold concentrations, rapidly increased the frequency and amplitude of these spontaneous contractions for a few seconds (Fig. 1j). Spontaneous contractions were usually only observed after stimulation by exogenous agonists or EFS and were not completely abolished by TTX.

Immunohistochemistry

The $P2X_1$ receptor expression was observed on the smooth muscle membranes in the outer longitudinal and middle circular muscle layers. Minimal expression was observed on the inner longitudinal layer (Fig. 3a). At higher magnification, it appeared that the $P2X_1$ receptor expression was nonhomogenous both in terms of the number of smooth

FIGURE 1

Typical traces of responses to electrical field stimulation (EFS) in the presence and absence of antagonists and individual responses to agonists. Start of time scale indicates the start of nerve stimulation or application of agonist to organ bath. (**a**) Response to EFS at 100 V, 0.5 milliseconds, 32 Hz for 20 seconds in the absence of an antagonist. (**b**) Stimulation in the presence of prazosin (1 μM). (**c**) Stimulation in the presence of tetrodotoxin (1 μM). (**d**) Response to EFS in absence of antagonist. (**e**) Stimulation in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (30 μM). (**f**) Stimulation in the presence of both PPADS (30 μM) and prazosin (1 μM). (**g**) Response to noradrenaline (100 μM). (**h**) α,β-meATP (300 μM). (**i**) ATP (1 mM). (**j**) Spontaneous contraction and subsequent reaction after ATP (10 μM) is applied.



Banks. Contraction of human vas deferens. Fertil Steril 2006.

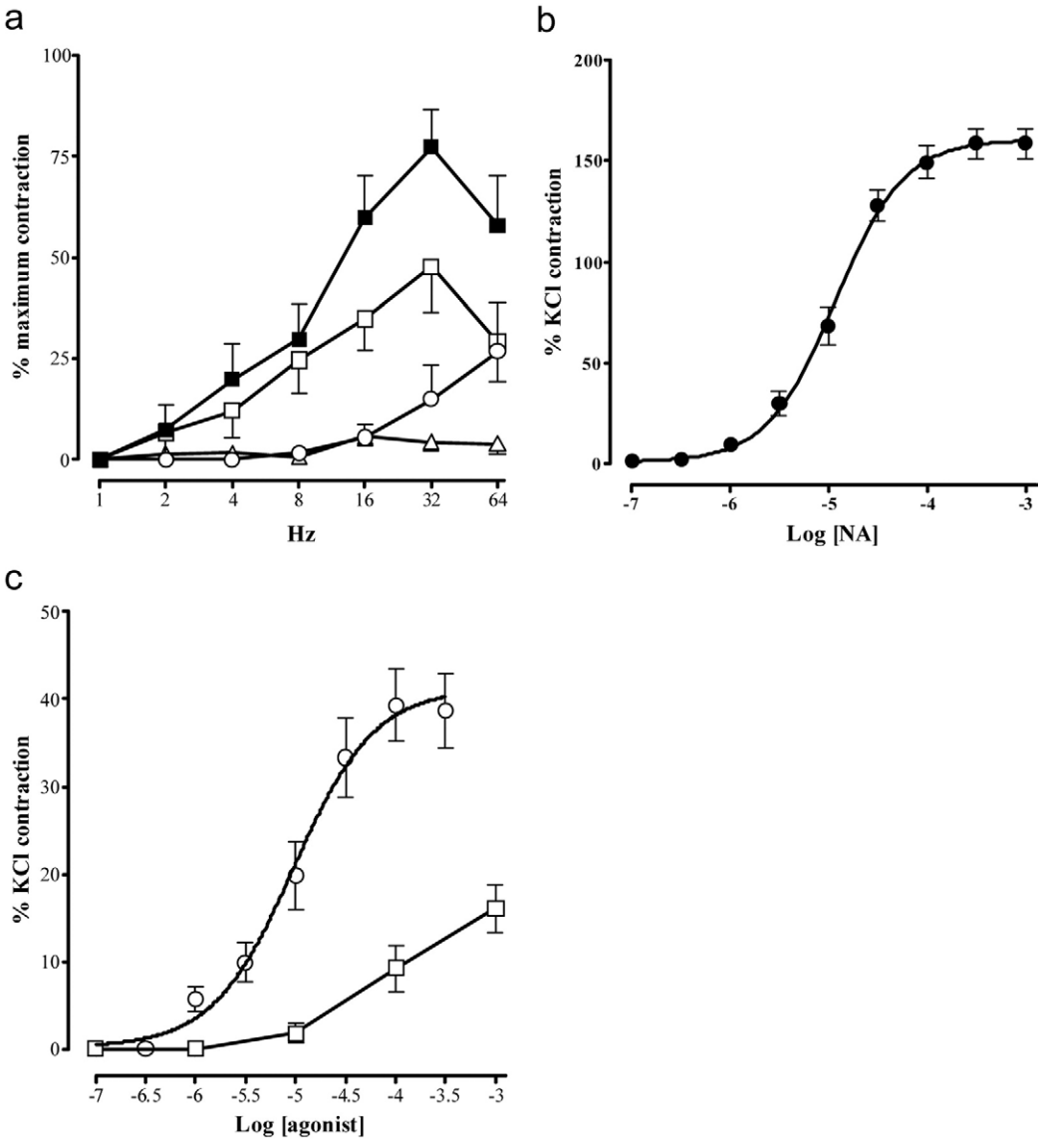
muscle cells on which it was expressed, and on single smooth muscle cells with expression being greater on some areas of the membrane (see Fig. 3b). Expression of $P2X_2$ receptor was also noted in a similar pattern, but more diffusely distributed and more often localized intracellularly (Fig. 4c,d). No expression of other P2X receptor subtypes was observed on the smooth muscle. In control studies, no expression was observed in the absence of the primary antibody or after preadsorption of the primary antibody with its cognate peptide.

DISCUSSION

The contraction of vas deferens from mammals such as the rat, rabbit, and guinea pig in response to sympathetic nerve stimulation has been shown to be biphasic (4, 14, 15, 29), ATP inducing the initial fast phase and NA the later slower phase (15). In contrast, the human vas deferens contracts to sympathetic nerve stimulation in a slower monophasic manner. At higher frequencies, and with higher concentrations of NA, suprathreshold rhythmic contractions were superimposed.

FIGURE 2

Frequency– and concentration–response curves in the human vas deferens. (**a**) Frequency–response curves of control contraction (■), then in the presence of pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (30 μM) (□), or prazosin (1 μM) (○), and then both PPADS (30 μM) and prazosin (1 μM) (△). The contractions have been corrected for the tetrodotoxin-insensitive component. (**b**) Concentration–response curve to noradrenaline (NA) (0.05–300 μM). (**c**) Concentration–response curves to α,β-meATP (0.1–300 μM) (○), and ATP (0.1 μM-1 mM) (□). All symbols show mean ± SEM (unless masked by the symbol), as a percentage of the maximum contraction (**a**) or as a percentage of the KCl contraction (**b** and **c**).

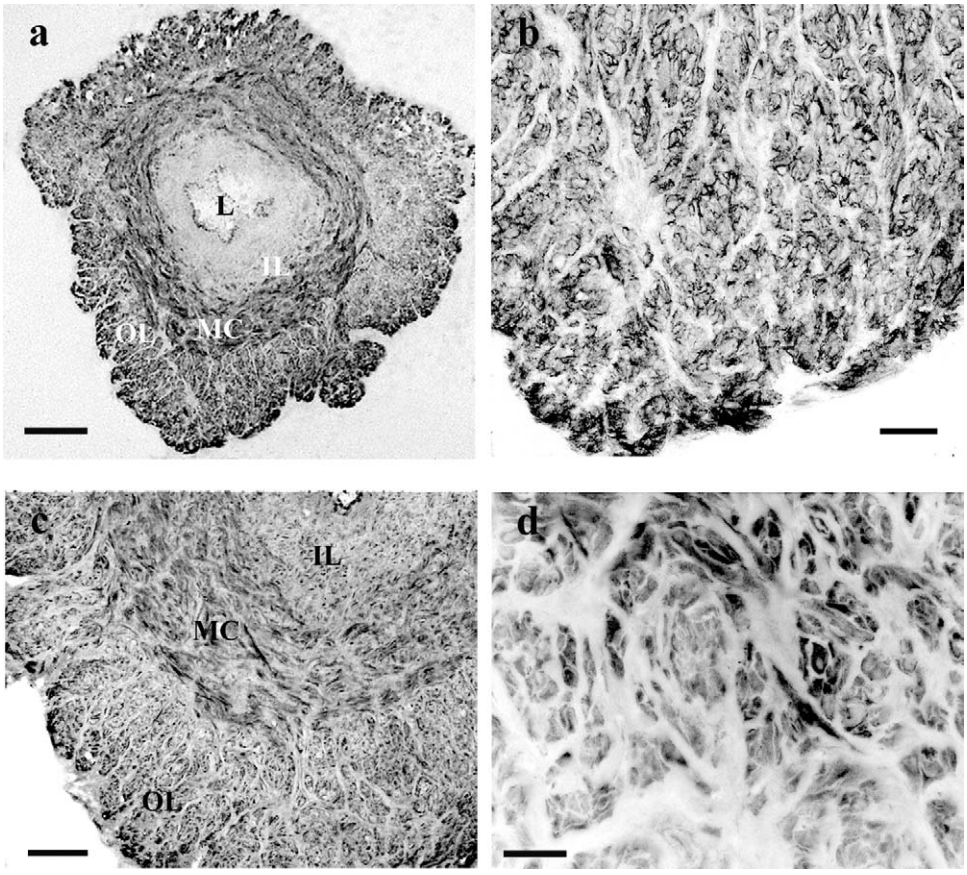


Banks. Contraction of human vas deferens. Fertil Steril 2006.

Maximal, nerve-mediated contractions, in the presence of PPADS were reduced by 40%. This corresponds to the purinergic component of neurogenically induced vas deferens smooth muscle contraction. The purinergic antagonism was much greater at the higher frequencies, with a corresponding reduction in adrenergic antagonism. This would suggest a differential in the amount of each neurotransmitter co-released from sympathetic nerves depending on the extent of stimulation. We found that prazosin inhibited the contraction by more than 80%, confirming that the adrenergic component is proportionally dominant. The relative adrenergic and purinergic components amounted to more than 100%, implying that synergism existed. Synergism between ATP and NA in the vas deferens of other animals has previously been shown (30–33).

FIGURE 3

Photomicrographs demonstrating $P2X_1$ and $P2X_2$ receptor immunolocalization on smooth muscle of human vas deferens. (**a**) Photomicrograph demonstrating $P2X_1$ receptor localization on outer longitudinal (OL) and middle circular (MC) smooth muscle layers. $P2X_1$ receptor expression was not observed on the inner longitudinal (IL) layer. The lumen is denoted by L. Scale bar, 250 μm. (**b**) Photomicrograph demonstrating $P2X_1$ receptor localization on smooth muscle membranes. Scale bar, 50 μm. (**c**) Photomicrograph demonstrating diffuse $P2X_2$ receptor expression on smooth muscle of the outer longitudinal (OL) and middle circular (MC) smooth muscle layers. Scale bar, 125 μm. (**d**) Photomicrograph demonstrating $P2X_2$ receptor expression in the cytoplasm of smooth muscle. Scale bar, 25 μm.



Banks. Contraction of human vas deferens. Fertil Steril 2006.

The $P2X_1$ receptor has been shown to be present on the vas deferens smooth muscle membrane in the mouse and rat by immunohistochemistry (18, 20). The α,β-meATP is specific for the $P2X_1$ and $P2X_3$ receptor subtypes (12). The $P2X_3$ receptor has been principally demonstrated on sensory nerves, and is not thought to be involved in smooth muscle contraction (34). The human vas deferens was contractile to α,β-meATP in a concentration-dependent manner. This would suggest that the functional receptor on smooth muscle in human vas deferens is also the $P2X_1$ subtype.

The pharmacological evidence was further supported by the immunohistochemical demonstration of $P2X_1$, but not $P2X_3$, receptor expression on the outer longitudinal and middle circular layers of smooth muscle of the human vas deferens. Expression was greatest on the outermost smooth muscle. The $P2X_2$ receptor expression was also demonstrated on the outer longitudinal and middle circular layers of smooth muscle cells. Previous investigators have demonstrated a similar pattern of expression in the rat vas deferens (16, 35). In the human vas deferens, sympathetic nerves run in the adventia and then spread into the muscularis forming an intramural network and a submucosal plexus staining acetylcholinesterase has also been demonstrated (36). It has been shown that in the human vas deferens, the ratio of nerve varicosities to smooth muscle is of the order of 1:30, whereas in the rat the ratio is as much as 1:1 (37). A previous study demonstrated that large clusters of $P2X_1$ receptors are present on smooth muscle opposite varicosities (17), whereas small clusters are not localized near varicosities. On this basis, in

human vas deferens one would expect fewer clusters of $P2X_1$ receptors than in the rat, which may explain the relative paucity of staining in the human vas deferens compared to the rat vas deferens. It would also explain the relatively greater $P2X_1$ receptor expression on the outer smooth muscle layer, as the nerve density is greater in the outer smooth muscle layer. The purinergic component of vas deferens contraction from the rat, rabbit, and guinea pig is greater at the prostatic end of the vas deferens compared to the epididymal end (38). If the human vas deferens is comparable, then greater expression would be expected in more proximal sections of vas deferens. In this study it was not possible to collect proximal vas deferens specimens to examine the possibility.

A previous study on the human vas deferens found that both adenosine and ATP inhibited responses to field stimulation by about 30% (25). The observed reduction in contraction was thought to be due to the P1 receptor-mediated prejunctional action of adenosine, arising from the ectoenzymatic breakdown of ATP. A more recent study of the rat vas deferens demonstrated facilitation of nerve-mediated NA release by stimulation of P2X receptors, and inhibition of NA release after stimulation of P2Y receptors, with no discernable P_1 receptor effect (39).

The tubular structure and three-layered smooth muscle arrangement of the vas deferens suggest that it contracts in a peristaltic fashion; however, objective evidence for this is lacking. Kimura et al. (40) demonstrated in dogs, that after hypogastric nerve stimulation, the epididymal section of vas deferens contracted, followed by the prostatic section 5–10 seconds later. Radiographic studies in rabbits have demonstrated that dye injected at the vas–epididymal junction is moved both toward the urethra, and back into the epididymis during sexual rest. Sexual stimulation rapidly moved dye toward the urethra for ejaculation, but residual dye was retrogradely transported back to the caudal epididymis (41). In our study, both spontaneous and neurogenic-induced contractions of the human vas deferens were demonstrated. Autonomic-induced contractions were far more forceful and are probably responsible for the main propulsion of spermatozoa through the vas deferens, presumably after sexual arousal. Spontaneous contractions may be responsible for the slow antegrade and retrograde movement of sperm seen in sexual rest, assuming that sperm movement in man mirrors that of the rabbit.

At present it is unclear exactly when the vas deferens of either laboratory animals or man contracts in physiological conditions. It is certainly thought to contract during ejaculation, but objective evidence for the timing and frequency of contraction is lacking. In the majority of preparations, spontaneous contractile activity was observed at a mean frequency of 100 per hour. In keeping with previous observations spontaneous activity was only observed after initial stimulation (24). Consistent with other investigators, we found a small part of the evoked response of the human vas deferens to be TTX-insensitive (24, 42).

The TTX reduced the frequency and amplitude of these spontaneous contractions, but did not abolish them completely, suggesting that inherent myogenic contractions occur in the vas deferens, particularly after stimulation. Exogenously applied ATP potentiated both the frequency and amplitude of spontaneous contractions. Studies have claimed dense expression of $P2X_2$ receptors on the lamina propria of the mouse, rat, and guinea pig vas deferens (35); however, their role has yet to be elucidated. Evidence is emerging for a possible action on the pacemaker cells of the myenteric plexus of the intestine where it is expressed on the interstitial cells of Cajal (43). Diffuse expression of $P2X_2$ receptors on smooth muscle was noted. If the $P2X_2$ receptor does have a pacemaker effect, then it may be that the enhancement observed with ATP is due to stimulation of the $P2X_2$ receptor.

This study has demonstrated that the human vas deferens smooth muscle is contractile to adrenergic and purinergic agonists, the adrenergic system being functionally dominant. However, purinergic co-transmission has also been shown to be functionally significant. The $P2X_1$ and $P2X_2$ receptor expression has been demonstrated on human vas deferens smooth muscle. In keeping with other species examined the functional receptor in smooth muscle contraction is the $P2X_1$ subtype, although $P2X_2$ receptors may have a role in regulation of spontaneous contractions. It is a possibility that by analogy with the human bladder that the ATP component of autonomic contraction is increased in pathological conditions (5, 7). The possibility of $P2X_1$ antagonists having a contraceptive role (44) remains an exciting future development. Alternatively adrenergic and purinergic stimulation may be indicated in the treatment of idiopathic oligospermia as suggested by two small case series, in which ephedrine or pseudoephedrine was used to stimulate aperistaltic vas deferens (45, 46).

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