BIOLOGY OF REPRODUCTION **74,** 473–480 (2006)
Published online before print 9 November 2005.
DOI 10.1095/biolreprod.105.044602

Smooth Muscle and Purinergic Contraction of the Human, Rabbit, Rat, and Mouse Testicular Capsule[1]

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ABSTRACT

The smooth-muscle cells of the testicular capsule (tunica albuginea) of man, rat, and mouse were examined by electron microscopy. They were characteristically flattened, elongated, branching cells and diffusely incorporated into the collagenous matrix and did not form a compact muscle layer. Contractile and synthetic smooth-muscle cell phenotypes were identified. Nerve varicosities in close apposition to smooth muscle were seen in human tissue. Contractions induced by adenosine 5′-triphosphate (ATP), alpha, beta-methylene ATP, noradrenaline (NA), acetylcholine (ACh), and electrical field stimulation (EFS) of autonomic nerves were investigated. Nerve-mediated responses of the rabbit and human tunica albuginea were recorded. The EFS-induced human responses were completely abolished by prazosin. In the rabbit, EFS-induced contractile responses were reduced by pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid by 36% and by prazosin by 77%. Both antagonists together almost completely abolished all EFS-induced contractions. The human tunica albuginea was contracted by NA, ATP, and alpha, beta-methylene ATP, but not by ACh. The rabbit and rat tunica albuginea were contracted by NA, ATP, alpha, beta-methylene ATP, and ACh. The mouse tunica albuginea was contracted by ACh, ATP, and alpha, beta-methylene ATP, but relaxed to NA. Immunohistochemical studies showed that $P2X_1$ (also known as P2RX1) and $P2X_2$ (also known as P2RX2) receptors were expressed on the smooth muscle of the rodent testicular capsule, expression being less pronounced in man. The testicular capsule of the rat, mouse, rabbit, and man all contain contractile smooth muscle. ATP, released as a cotransmitter from sympathetic nerves, can stimulate the contraction of rabbit smooth muscle. Human, rat, and mouse testicular smooth muscle demonstrated purinergic responsiveness, probably mediated through the $P2X_1$ and/or $P2X_2$ receptors.

male reproductive tract, male sexual function, neurotransmitters, testis

INTRODUCTION

There is increasing evidence for falling sperm counts in man [1, 2]. Despite the World Health Organization standardization of semen-analysis parameters [3], it is difficult to define normal in the multiple parameters of semen analysis, and there is widespread variation in laboratory measurement of semen analysis [4]. Semen analysis is the end product of sperm production, transport, and ejaculation through the genitourinary system. A reduction in sperm numbers may reflect reduced transportation through the genital tract rather than reduced production. For instance, in young, healthy volunteers who gave a minimum of five samples in the course of a year, sperm concentration varied by an average of 4.8-fold, with the maximum difference being 17.2-fold [5]. There is no clinical or laboratory measure of sperm transport failure and exceedingly limited objective evidence for the mechanisms of sperm transport, with much evidence implied from relatively dated anatomical studies. It may be, therefore, that a significant number of patients labeled as idiopathic oligospermia have a degree of failure of the transport process rather than spermatogenesis. This subset of infertile patients is potentially amenable to stimulation of genital smooth muscle with either adrenergic or purinergic stimuli, should such an agent become available.

Our knowledge of the basic contractile mechanisms and the frequency of such contractions in propelling sperm through the genital tract are surprisingly deficient. It is postulated that contraction of the tunica albuginea is one mechanism propelling sperm from the seminiferous tubules into the head of the epididymis [6]. Since the demonstration of contractile smooth muscle in the tunica albuginea in the late 1960s, our understanding of autonomic signaling has advanced with the acceptance of purinergic cotransmission. The biphasic nature of vas deferens contraction was instrumental in this acceptance [7], and the importance of purinergic signaling in the contraction of genital smooth muscle has been confirmed by $P2X_1$ (also known as P2RX1) receptor-deficient mice being infertile due to oligospermia, secondary to reduced vas deferens contraction [8]. Contraction of the human vas deferens has recently been shown to have a significant purinergic component [9]. The testicular capsule can be considered to be the most proximal part of the vas deferens and, in conjunction with the seminiferous tubules, constitutes the origin of the genital tract. Contraction of the tunica albuginea may be essential in moving sperm out of the testis and it is important to our understanding of sperm transport that the contractile mechanisms governing the smooth-muscle contraction are characterized.

Purinergic cotransmission is now well established for the contractions produced by sympathetic nerves in vas deferens [10] but has not been examined in the testis. Knowledge of purinergic signaling is rapidly advancing and appears to be implicated in smooth-muscle dysfunction, e.g., detrusor overactivity [11]. Furthermore, the absence of $P2X_1$ receptors in transgenic mice resulted in infertility, probably due to vas deferens dysfunction [8]. As the tunica albuginea can be

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Received: 13 June 2005.
First decision: 20 July 2005.
Accepted: 8 November 2005.

ISSN: 0006-3363. http://www.biolreprod.org

considered as a proximal extension of the vas deferens and epididymis, tunica albuginea smooth-muscle dysfunction may be implicated in infertility. As such, the following comparative study was carried out to examine the testicular capsule smooth-muscle elements from man, rabbit, rat, and mouse. The rat was chosen, as the P2X antibodies were developed in this species. The mouse was chosen to characterize this species in preparation for studies on gene-deleted mice, and the tunica albuginea contraction has not been characterized previously in this species. The rabbit was included, as it is documented to have a relatively high smooth-muscle content. Man was studied to give clinical relevance to the comparative study. The role of purinergic cotransmission in the contraction of the tunica albuginea smooth muscle was examined by electrical field stimulation of autonomic nerves and the exogenous application of purinergic and autonomic agonists. The distribution of P2X receptors was examined by immunohistochemistry.

MATERIALS AND METHODS

Principles of good laboratory animal care were followed and animals were killed in compliance with Schedule 1 of the Home Office (U.K.) regulations. Animals were obtained from in-house stock. Human testes were obtained from patients undergoing orchidectomy for either prostate cancer or gender reassignment. All patients gave prior informed consent, and the local ethics committee approved the study.

Histology

Human tissue. Sections of capsule from the middle area of the testis were mounted at different orientations. The tissue was embedded in Tissue Tek OCT compound (Sakura, Zoederwoude, The Netherlands) and frozen in isopentane precooled in 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 min before use.

Animal tissue. Whole or half testes were prepared in the same way as for human tissue (see above). Whole mounts of the testicular capsule were also examined.

Primary antibodies. The immunogens used were peptides corresponding to 15 receptor type-specific amino acids in the C-terminal region of the human and rat $P2X_{1-7}$ receptors (also known as P2RX1–7) (Roche Bioscience, Palo Alto, CA). The synthetic peptides were covalently linked to limpet hemocyanin and the conjugate was administered to rabbits at monthly intervals (performed by Research Genetics, Huntsville, AL). Immunoglobulin G (IgG) fractions were isolated from the immune and preimmune sera for the seven P2X receptors using the method of Harboe and Ingild [12]. The specificity of the antibodies was verified by immunoblotting as previously described. $P2X_1$ is known to be identical in the rat and human and other receptors show high levels of conservation between species [13].

The avidin-biotin technique as described by Llewellyn-Smith was used [14, 15]. Briefly, the slides were fixed in 4% formaldehyde and 0.2% of a saturated picric acid solution in 0.1 M phosphate buffer for 2 min. To inactivate endogenous peroxidase, the sections were then treated with 50% methanol containing 0.4% hydrogen peroxide for 10 min. Nonspecific binding sites were blocked by incubating with 10% normal horse serum (NHS) in phosphate buffered saline containing 0.05% thimerosal (Merthiolate) for 20 min. The P2X receptor antibodies were diluted to 2.5–5 μg/ml (determined by prior titration) with 10% NHS and the sections were incubated with primary antibodies overnight at room temperature. The secondary antibody was a biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Luton, U.K.) diluted 1:500 and incubated for 1 h. Sections underwent a further incubation with extravidin peroxidase (Sigma Chemical Co., Poole, U.K.) at 1:1000 for 1 h.

The reaction product was visualized using the nickel-diaminobenzidine enhancement technique. The specimens were dehydrated in xylene and mounted in a xylene-based mountant. Controls were performed with preimmune IgG antibodies adsorbed with their homologous peptides and in the absence of primary antibodies. The results were documented using the Edge R400 high-definition light microscope (Edge Scientific Instruments, Santa Monica, CA.). Pictures were stored using digital camera technology (Leica 2000; Leica, Heerbrugg, Switzerland) and printed using Adobe Photoshop (6.0 edition).

Electron microscopy. Fresh tissue was fixed in 4% paraformaldehyde and 0.1% glutaraldehyde. Sections were cut on an Oxford vibratome (Lancer) and collected in serial order in 0.1 M phosphate buffer. They were subsequently dehydrated in ethanol, cleared in propylene oxide, and embedded in araldite between two sheets of Melanex (ICI, Middlesborough, U.K.). Semithin (1-μm) sections were cut with glass knives and stained with toludine blue. Thin sections (70 nm) were cut with a diamond knife on a Reichert Ultracut ultramicrotome (Leica CM 3050; Leica, Nussloch, Switzerland) and stained in the same way. The sections were collected on copper-mesh grids coated with a thin Formvar film (Agar Scientific, Stansted, U.K.), counterstained with lead citrate, and viewed in a JEOL 1010 transmission electron microscope (JEOL Instruments, Akishima, Japan).

Pharmacology

General procedures. Adult male Sprague-Dawley rats (300–360 g; n = 5), mice (35–42 g; n = 5), and rabbits (3 kg; n = 3) were killed and the testes were immediately amputated and placed in modified Krebs solution. The tissues were then stripped of adhering epididymis, fat, and connective tissue and prepared for isolated organ bath recordings. Mouse and rat testes were opened on their antihilar border, and the entire testicular capsule was gently peeled off the seminiferous tubules. In this way, a whole capsular preparation was obtained. Silk ligatures were attached to both ends of the capsule, one end was attached to a rigid support and the other to a FT-03C force-displacement transducer (Grass Instruments, Quincy, MA). For human and rabbit tissue, longitudinal strips of testicular capsule, approximately 15 × 2 mm, were dissected free from each testis. Strips for electrical field stimulation (EFS) were cut parallel to blood vessels. Strips were mounted in a similar manner to whole capsule preparations. Each tissue was suspended in a 10-ml organ bath containing continuously gassed (95% O_2/5% CO_2) modified Krebs solution of the following composition (mM): 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; pH 7.3. Experiments were carried out at 35 ± 1°C to replicate scrotal temperature. Separate experiments were carried out to evaluate EFS and the action of exogenously applied agonists noradrenaline (NA), acetylcholine (ACh), adenosine 5′-triphosphate (ATP), and the slowly hydrolyzable ATP analog α,β-methylene ATP (α,β-meATP). Log concentration-response curves to individual aliquots of NA (10 nm–300 μM), α,β-meATP (100 nM–300 μM) and ATP (100 nM–1 mM) were recorded. Aliquots were given at least 10 min apart to avoid desensitization and the tissue was rinsed with at least 30 ml of Krebs solution between each aliquot. ACh (10 nm–300 μM) concentration-response curves were established by adding aliquots cumulatively once each increasing contraction had achieved a plateau. The contraction due to a standard concentration of 120 mM potassium chloride (KCl) was noted at the end of each experiment. All results were expressed as the percentage of the response to KCl.

Frequency-response curves. Nerve stimulation of the testicular capsule 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, NSW, Australia). An initial load of 1 g was applied to human and rabbit tissue, 0.5 g to rat tissue, and 0.2 g to mouse tissue, which were then allowed to equilibrate for not less than 45 min before the start of the experiment. Tissue strips were subjected to EFS at 100 V, 0.3- to 0.5-ms pulse duration, 2–64 Hz, for 30–60 sec every 5 min. For those strips with consistent contractions, the EFS was repeated in the presence the α_1 adrenoceptor antagonist prazosin 1 μM and also the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) at a concentration of 30 μM. All results were expressed as a percentage of the KCl (120 mM) response.

Drugs used. ATP, α,β-meATP (lithium salt), NA, ACh, and KCl were obtained from Sigma. Stock solutions were prepared in distilled water or ascorbic acid for NA. The volume added to the organ bath to produce the final concentration was not in excess of 100 μl.

Statistical analysis. Contractile responses were expressed as mean percentage of the maximum response to KCl (120 mM) ± SEM. As the concentration-response curve to ATP did not reach a maximum, it was not possible to calculate the EC_{50} (median effective dose) concentration. Statistical analysis was carried out using Graph Pad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). Concentration-response curves were analyzed with a two-way analysis of variance (ANOVA) followed by a post hoc test (Bonferroni test). The hypothesis was rejected if $P > 0.05$.

RESULTS

Electron Microscopy

In the three species examined (man, rat, mouse), two layers of testicular capsule were identified. The main substance of the capsule was the tunica albuginea, consisting of dense connective tissue and smooth-muscle cells (Fig. 1A). An inner layer, the tunica vasculosa, was identified, which contained loose connective tissue incorporating the vasculature and nerve bundles. Previous investigators identified an outer mesothelial layer, one cell in thickness, termed the tunica vaginalis visceralis. This was not visualized in our preparations, though it has been reported that this is often lost in tissue preparation [16].

The three species examined demonstrated a similar distribution of smooth-muscle cells within the tunica albuginea. Flattened branching cells were loosely arranged in sheets of a single cell in thickness, with intervening collagenous connective tissue. These sheets were closer together on the inner aspect of the tunica albuginea, having less intervening collagen between them, but did not form an anatomically distinct muscle layer within the tunica albuginea (see Fig. 1B). The smooth-muscle cells were mostly arranged in a longitudinal pattern, though those of the human tunica albuginea were the least organized of the three species examined. The smooth-muscle cell appearance varied depending on its position within the tunica albuginea. The cells of the outer aspect largely resembled the synthetic smooth-muscle phenotype, having a greater cytoplasm-to-nuclear ratio, greater amounts of Golgi apparatus, fewer dense bodies, and fewer myofilaments. In contrast, the cells of the inner aspect had a more classical appearance of the contractile smooth-muscle phenotype (Fig. 1, C and E). These cells were flatter, had central cylindrical nuclei, numerous dense bodies among myofilaments, dense plaques adherent to the cell membrane, micropinocytotic vesicles, and distinct basal laminae except at regions of cell-cell contact. Between smooth-muscle cells, large amounts of collagen and, to a lesser extent, elastin were present.

In the human tunica albuginea proper, nerves were seen that were not associated with blood vessels. Axon varicosities containing vesicles, in association with retracted Schwann cells, were seen in immediate apposition to smooth-muscle cells, strongly suggesting functional innervation of smooth muscle (Fig. 1, C and D). In contrast, in the mouse and the rat, no nerve fibers were identified within the tunica albuginea. In all three species examined, some specialized junctions between smooth-muscle cells were seen. These consisted of close apposition between cell membranes of smooth-muscle cell processes (Fig. 1E). However, these junctions were not examined at sufficiently high resolution to identify them as classical gap junctions. Nerves were predominately associated with blood vessels in the tunica vasculosa (Fig. 1F).

Light Microscopy/Immunohistochemistry

Only minimal $P2X_1$ receptor immunoreactivity was observed in the capsule of the human testis. Greater immunoreactivity was observed in the capsule of the rat and the mouse (Fig. 2A). $P2X_2$ receptor immunoreactivity was observed in all three species (Fig. 2C). Cross-species reactivity with P2X antibodies has previously been demonstrated in the mouse [17]. $P2X_3$ receptors were expressed in a few variable punctate places in the human tunica albuginea, but minimally so in the other species examined. No smooth-muscle expression of $P2X_{4-7}$ receptors was demonstrated in any of the species examined, although expression within the germ cells was noted to confirm antibody activity (data not shown). No immunoprecipitation was observed with control studies following preabsorption of the primary antibody with the relative P2X receptor peptides (Fig. 2, B and D).

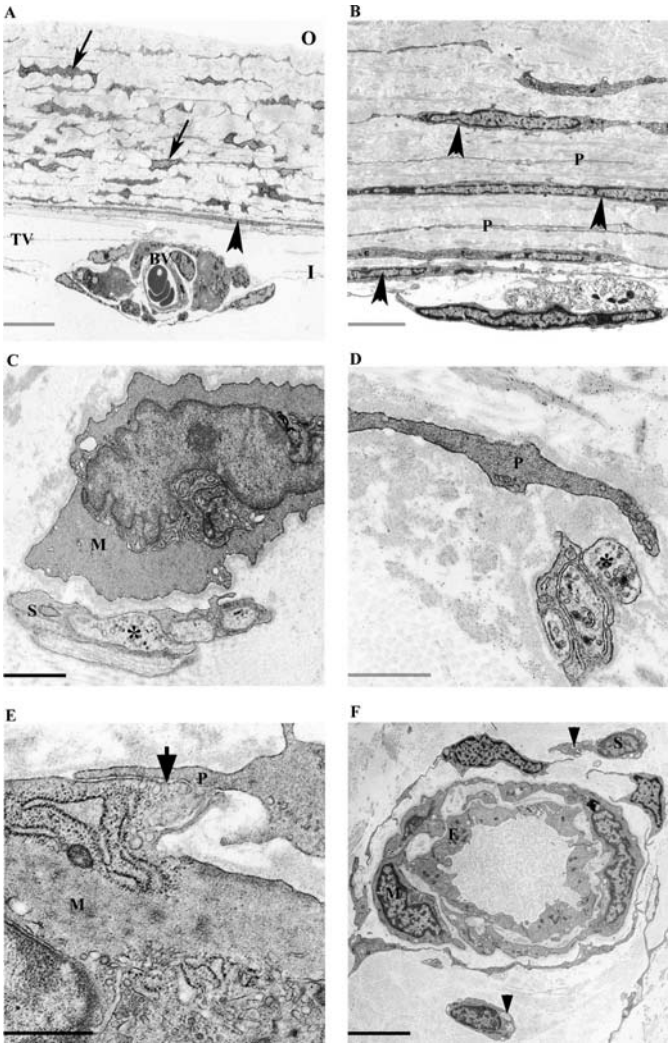

FIG. 1. **A**) Electron micrographs demonstrating the longitudinally orientated smooth-muscle arrangement in the tunica albuginea of the rat testis. Smooth-muscle cells are distributed singularly, embedded in connective tissue. They become closer to each other from the outer (O) to the inner (I) aspect of the sheet. The tunica vasculosa (TV) can be seen containing a blood vessel (BV). Arrows indicate the smooth-muscle cell bodies of the secretory phenotype smooth muscle. An arrowhead indicates a thinner and flatter contractile phenotype smooth-muscle cell on the inner aspect of the tunica albuginea. Bar = 10 μM. **B**) Electron micrograph of the mouse tunica albuginea demonstrating the increasing density of the flatter contractile smooth-muscle cells (arrowheads) on the inner aspect of the tunica albuginea. Processes of smooth-muscle cells (P) are interspersed between cell bodies. Bar = 2 μM. **C**) Electron micrograph demonstrating a smooth-muscle cell (M) in immediate apposition to a vesicle-containing axon profile (*), with no Schwann cell (S) process intervening. Bar = 1 μM. **D**) Electron micrograph demonstrating a smooth-muscle cell process (P) close to an axon profile (*), free of intervening Schwann cell process. Bar = 1 μM. **E**) Electron micrograph demonstrating an area of cell-cell contact (thick arrow) between a human smooth-muscle cell (M) and the process (P) of another smooth-muscle cell. Bar = 1 μM. **F**) Electron micrograph showing a typical blood vessel and associated nerve bundles (arrowhead) together with Schwann cells (S) running together toward the deeper region of the human tunica albuginea. E, Endothelial cell, M, smooth-muscle cell, S, Schwann cell. Bar = 2.5 μM.

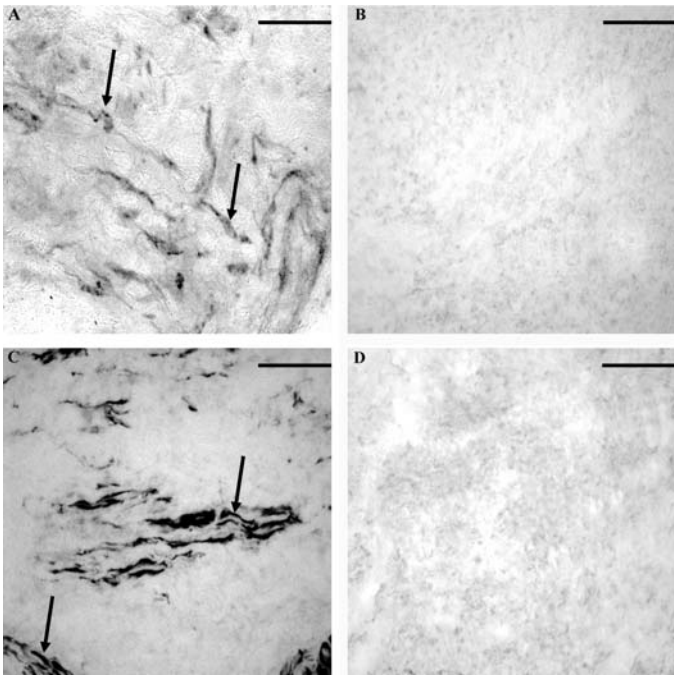

FIG. 2. Light microscopy/immunohistochemistry of tunica albuginea. **A**) This transverse section shows some immunoreactivity to the $P2X_1$ receptor subtype in the tunica albuginea of the rat. Arrows indicate smooth-muscle cells. Bar = 25 μM. **B**) Transverse section of the tunica albuginea of the rat. Preabsorption control with $P2X_1$ receptor peptide; no positive immunoreactivity was observed. Bar = 25 μm. **C**) This longitudinal section shows $P2X_2$ receptor immunoreactivity on human tunica albuginea smooth muscle (arrows). Bar = 100 μM. **D**) Longitudinal section of human tunica albuginea. Preabsorption control with $P2X_2$ receptor peptide; no positive immunoreactivity was observed. Bar = 25 μm.

Pharmacology

Strips of rabbit tunica albuginea were contractile to EFS of autonomic nerves in a frequency-dependent manner, with peak contraction occurring at 16 Hz. Contractions were biphasic, with an initial rapid phase followed by a longer plateau phase that took up to 30 sec to reach a maximal contraction. Subsequent EFS in the presence of purinergic antagonist PPADS (30 μM) reduced the maximal contraction by 36%. EFS in the presence of the adrenergic antagonist prazosin (1 μM) reduced the maximal contraction by 77%. When both antagonists were used together, EFS-induced contraction was almost completely abolished (Fig. 3A). Some strips of human tunica albuginea were contractile to EFS of autonomic nerves in a frequency-dependent manner, but contractions were less repeatable and the maximal contraction occurred at 32 Hz. Contractions were completely abolished by prazosin (Fig. 3B). The effect of PPADS was minimal or absent. In contrast, the rat and mouse tunica albuginea preparations were not contractile to EFS of autonomic nerves.

Strips of human tunica albuginea were contractile to exogenously applied NA, ATP, and α,β-meATP in a concentration-dependent manner, although contractions were recorded only to the higher doses of ATP tested (Fig. 4, A and B). The orientation of the strip did not influence the contractions. The strongest contractions were to NA, with the maximal NA contraction approximately six times stronger than that of the maximal α,β-meATP contraction. Respective EC_{50} values were NA 2.6 μM and α,β-meATP 5.6 μM. Contractions to ATP did not reach a maximum and so an EC_{50} could not be calculated. No contraction or relaxation was recorded to ACh.

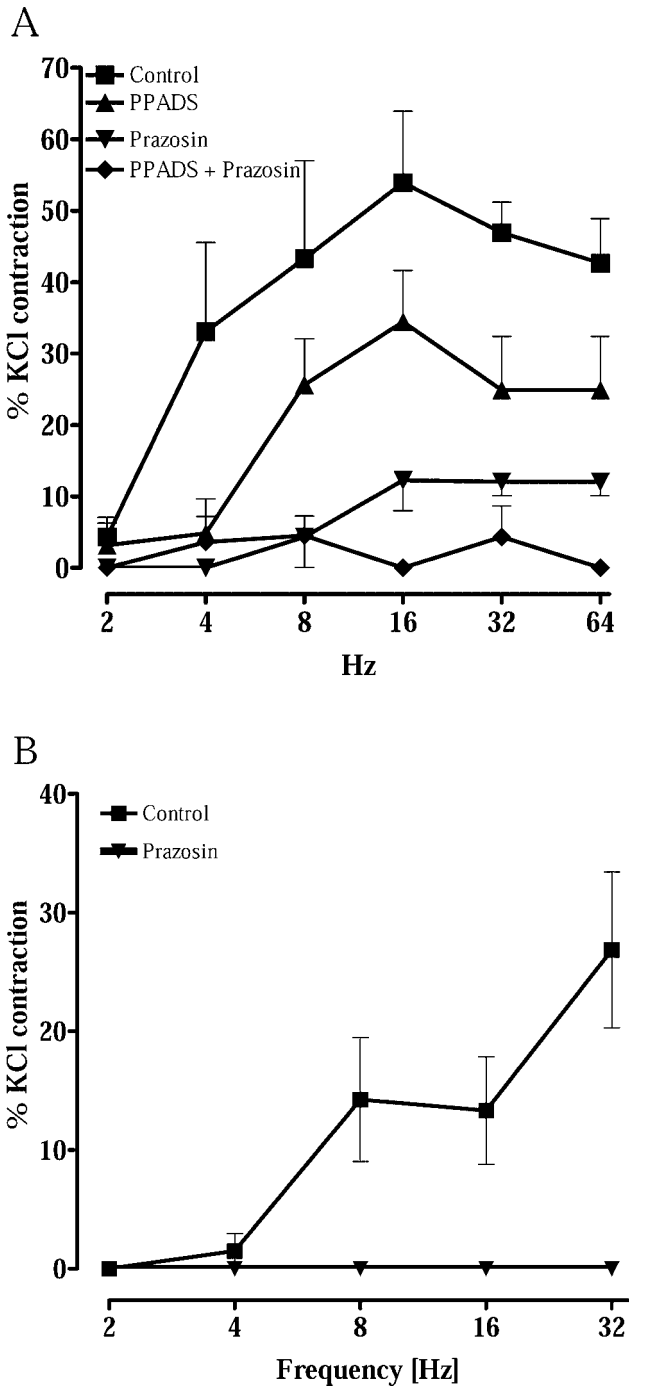

FIG. 3. Frequency-response curves showing frequency-dependent contraction of the rabbit and human tunica albuginea to EFS. All symbols show mean percentage of contraction to KCl (120 mM) ± SEM (n). **A**) Contraction of the rabbit tunica albuginea to EFS (100 V, 0.3 msec, 2–64 Hz, 15 sec). In the presence of PPADS (30 μM). In the presence of prazosin (1 μM), and in the presence of both PPADS (30 μM) and prazosin (1 μM) (n = 3). **B**) Contraction of the human tunica albuginea to EFS (100 V, 0.3 msec, 2–32 Hz, 30 sec) (n = 6). Contractions were completely antagonized by prazosin (1 μM).

Strips of rabbit tunica albuginea contracted to exogenously applied NA, ACh, ATP, and α,β-meATP in a concentration-dependent manner. The strongest observed contractions were to NA, then to ACh, with ATP and α,β-meATP being similar. Contractions to NA, ACh, ATP, and α,β-meATP did not reach a maximum and so EC_{50} values could not be calculated (Fig. 5A).

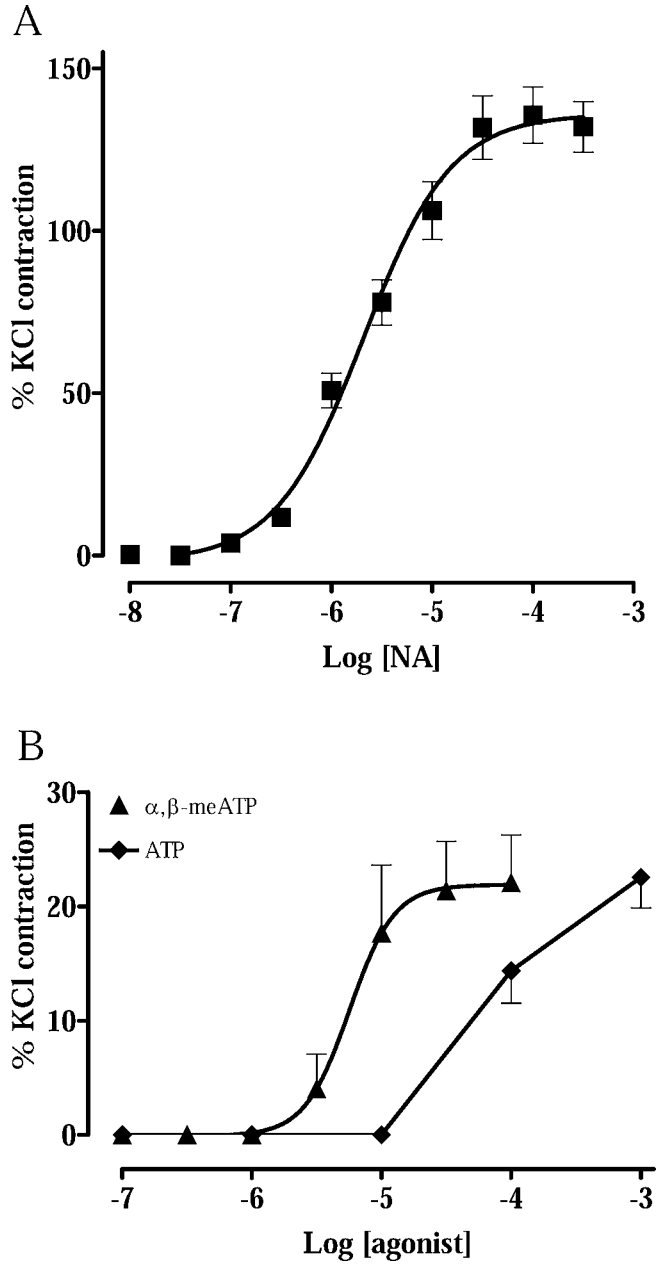

FIG. 4. Concentration-response curves for NA, ATP, and α,β-meATP on isolated strips of human tunica albuginea. All symbols show mean percentage of KCl contraction (120 mM) ± SEM (n) (unless masked by symbol). **A**) Concentration-response curve for NA (n = 7, strips = 11). **B**) Concentration-response curve for α,β-meATP (n = 7, strips = 11), and ATP (n = 12, strips = 16).

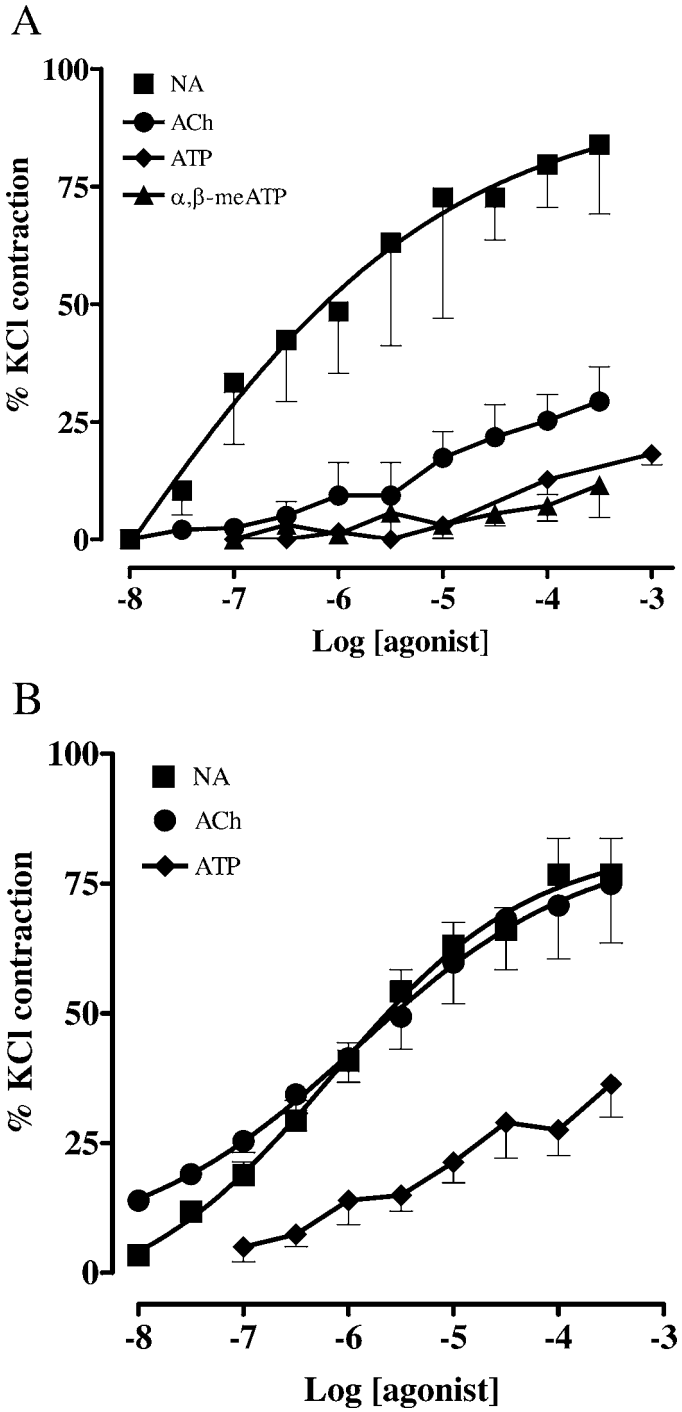

FIG. 5. Concentration-response curves for NA, ACh, ATP, and α,β-meATP on isolated strips of rabbit tunica albuginea and whole tunica albuginea preparations of the rat. All symbols show mean percentage of maximum KCl contraction ± SEM (n) (unless masked by symbol). **A**) Rabbit tunica albuginea. Concentration-response curve for NA, ACh, ATP (n = 3, strips = 3), and α,β-meATP (n = 2, strips = 2). **B**) Rat whole preparations of tunica albuginea. Concentration-response curve for NA, ACh, ATP (n = 6, preparations = 12).

Rat tunica albuginea preparations contracted to ACh, ATP, and NA in a concentration-dependent manner. The contractions to NA and ACh were very similar. Respective EC_{50} values were NA 0.68 μM, ACh 1.22 μM. No maximum was reached with ATP, so the EC_{50} could not be calculated (Fig. 5B). Contraction to α,β-meATP was recorded, but rapid desensitization occurred so that it was not possible to prepare a concentration-response curve.

The mouse tunica albuginea contracted in response to ACh in a concentration-dependant manner, giving an EC_{50} value of 0.58 μM. The capsule was also contractile in response to exogenous ATP; however, no maximum was reached (Fig. 6A). Contractions to α,β-meATP were recorded but rapid desensitization occurred, which prohibited a concentration-response curve being calculated. The whole capsule preparation, when precontracted with ACh at its EC_{50} concentration, was found to relax in response to the addition of NA in a concentration-dependent manner, giving an EC_{50} value of 10.2 μM (Fig. 6B).

The strips of rabbit testicular capsule demonstrated marked spontaneous activity with small contractions occurring at

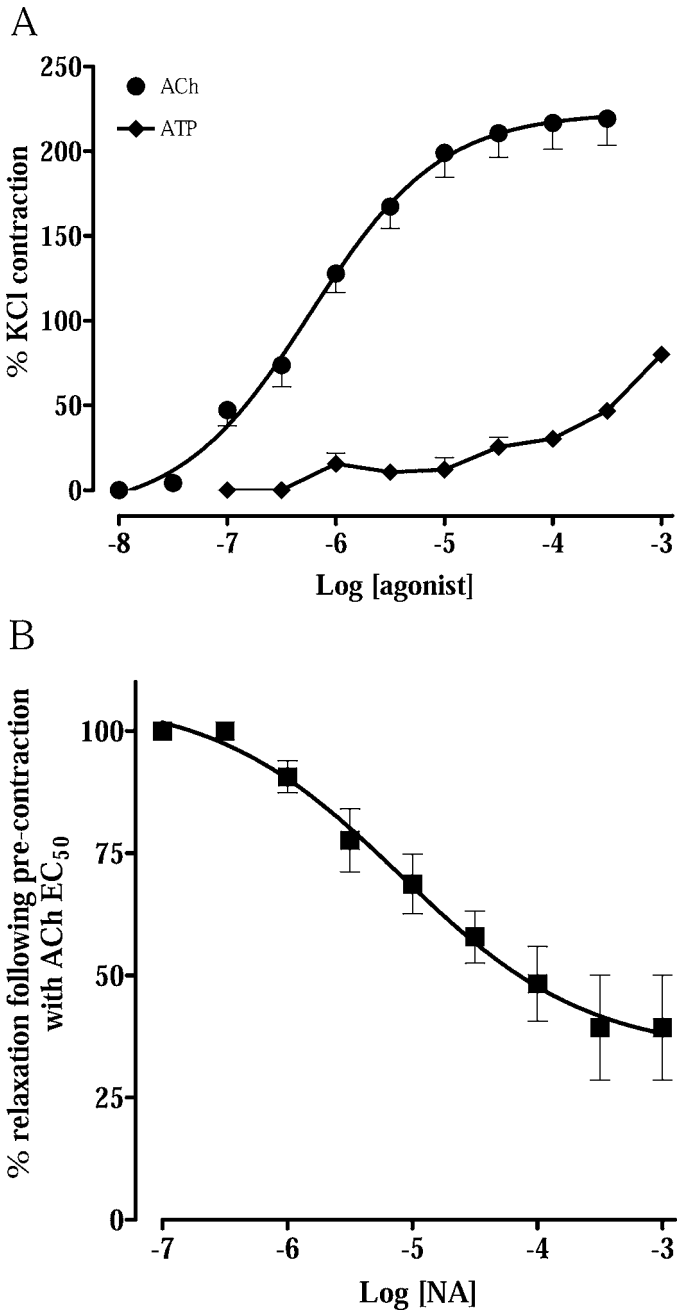

FIG. 6. **A**) Concentration-response curves for ACh and ATP on whole tunica albuginea preparations of the mouse. All symbols show mean percentage of maximum KCl contraction ± SEM (n) (unless masked by symbol) (n = 6, strips = 8). **B**) Relaxation concentration-response curves of whole preparations of mouse tunica albuginea to NA, following precontraction with ACh at the EC_{50} concentration. All symbols show mean remaining contraction expressed as percentage of contraction to ACh (EC_{50}) ± SEM (n) (unless masked by symbol) (n = 4, preparations = 4).

a rate of 140/h. Human testis strips also demonstrated some spontaneous activity, but only following stimulation with NA or ATP. Contractions were less consistent (5/9 preparations) and at a mean rate of 75 contractions/h.

DISCUSSION

Smooth-muscle cells were first demonstrated in the tunica albuginea of rabbit testes in 1967 [18]. Subsequently, smooth-muscle cells have been demonstrated in the tunica albuginea of several species, including man, rat, guinea pig, cat, dog, pig, cow, sheep, and horse [16, 19–23]. The extent and conformity of the smooth-muscle arrangement between species is not consistent, and only in the rabbit and man has a true layer been demonstrated, and indeed two layers perpendicular to each other have been described on the outer aspect of the rabbit tunica albuginea [23, 24]. The density of smooth-muscle cells in different areas of the tunica albuginea has been shown to be variable [18]. Interspecies variations exist and it has been suggested that more smooth-muscle cells are located at the poles and the posterior border of the testis in the rete testicular area [16]. In our own studies, we found no conclusive pattern or density of smooth-muscle cells in different areas of the testicular capsule. We found that the smooth-muscle cell phenotype progressively changed from a secretory phenotype on the outer aspect to a more contractile phenotype on the inner aspect of the tunica albuginea. This arrangement is largely in agreement with the findings of Middendorff, who confirmed the phenotypic change by immunohistochemistry [23]. However, we did not observe the inner smooth-muscle cells to be a distinct layer. This transition and arrangement was seen in all three species examined but was best demonstrated in the thin tunica albuginea of the mouse or rat, in which the entire testicular capsule could be observed in the same section. This observation may explain the mixed reports regarding the presence of true smooth-muscle cells as opposed to contractile myofibroblasts and is in keeping with the observation that a smooth-muscle cell is not a single entity but represents a heterogenous cell that is both contractile and synthetic at opposite ends of the spectrum [25].

The neuronal supply to the testis appears to vary markedly between species, with some immunohistochemical reports of dense networks covering the outer aspect of the tunica albuginea with nerves penetrating the substance of the testis and even directly innervating Leydig cells [26–28]. Other reports suggest the innervation is sparse and predominantly involved in vasomotor control and consequently thermoregulation [29, 30]. In the human testis, myelinated nerves penetrated the tunica albuginea, and nerve varicosities containing vesicles were seen in apposition to smooth-muscle cells, consistent with neurogenic innervation of smooth muscle. The strips of human tunica albuginea that did respond to EFS had all been cut either parallel or adjacent to a blood vessel that was subsequently removed. This would imply that the neurogenic innervation of the smooth muscle is related to nerves associated with the blood vessels. In keeping with other investigators, we found specialized junctions between smooth-muscle cells; these were fused areas between smooth-muscle cells, consistent with myogenic propagation of contraction, although no classical gap junctions were identified.

In 1969, Davis and Langford [31] first demonstrated that the rat testicular capsule contracted in response to NA and ACh. The present study demonstrated that the rabbit tunica albuginea was contractile to EFS of autonomic nerves. The P2 receptor antagonist PPADS reduced the contractile force by 36% and prazosin by 77%. In combination, virtually no contraction was detected. The response of the rabbit tunica albuginea to exogenous α,β-meATP was uncharacteristically lower than that to ATP, which is suggestive that some desensitization of $P2X_1$ receptors may have occurred, as seen in the rat and the mouse, or that $P2X_2$ receptors were involved. In the rabbit, the maximal contractile force induced by NA was approximately four times that of ATP or α,β-meATP. In contrast, the human tunica albuginea contraction was completely antagonized by prazosin and the maximal contractions induced by NA were

approximately seven times that of α,β-meATP. The purinergic neural component of human tunica albuginea contraction may be smaller and masked by the adrenergic component. The pattern of a relatively smaller purinergic component would be in keeping with other studies on purinergic smooth-muscle contraction of healthy human genitourinary smooth muscle [11, 32]. In keeping with previous findings [31], the rat tunica albuginea was contractile to both NA and ACh, although in our study, we found marginally greater contractility with NA as opposed to ACh. In all the species examined, we have shown for the first time that the tunica albuginea is also contracted by purinergic agonists. The mouse tunica albuginea demonstrated a different pharmacological contractile profile from that of the rat and rabbit, where the tunica albuginea contracted to ACh and ATP, but relaxed to NA.

In other tissues, where purinergic cotransmission is demonstrated, such as the vas deferens or bladder, the contraction is classically biphasic with an initial rapid phase being attributable to ATP and a second slower phase attributable to either NA or ACh, respectively [33–35]. The contraction of rabbit tunica albuginea to EFS was demonstrated to be biphasic. In the human tunica albuginea, where adrenergic transmission dominates, contraction to EFS was monophasic and contraction to exogenous agonists was slow, taking up to 1 min to reach maximum. This slow pattern was similar in the rat and the mouse.

ATP-induced smooth-muscle contractions have been studied in other tissues and it is established that contraction is mediated through the $P2X_1$ receptor. Alpha,β-meATP is selective for $P2X_1$ and $P2X_3$ receptors. $P2X_3$ receptors are largely expressed on sensory nerves involved in nociception [11, 36], which suggests that smooth-muscle contraction is largely mediated through the $P2X_1$ receptor subtype, with the exception of the rabbit, where contractions to α,β-meATP were substantially less than ATP and $P2X_2$ receptors may be involved. Immunohistochemical studies using the seven P2X receptor subtype antibodies weakly demonstrated the presence of $P2X_1$ receptors on the smooth-muscle membrane. There was also expression of $P2X_2$ receptors on smooth-muscle cells, although often located intracellularly. Staining of both $P2X_1$ and $P2X_2$ receptors was generally low when compared with other tissues, such as the vas deferens, bladder, and arteries. This is partly a reflection of the small smooth-muscle content within testes and the relatively small purinergic component, at least in healthy tissues.

Sperm contained within the testis lack forward progressive motility, and this is subsequently gained in the distal epididymis in man and rats [37–39]. A pressure gradient would move sperm out of the testis, and it is postulated that contraction of the testicular capsule and myoid cells of the seminiferous tubules may generate this [40–42]. Fluid production within the testis would also create pressure, and movement of the fluid would have the additional benefit of effectively carrying sperm out of the testis on an effluent tide. Movement of the ciliated cells of the ducti efferenti at the junction of the rete testis and caput epididymis was previously thought to move sperm into the epididymis, but this has been disproved due to the demonstration of normal numbers of sperm in the epididymis in patients with Kartageners syndrome, in which cilia are immobile [43]. Studies using radio-opaque sperm injected into the epididymis of rabbits have shown significant antegrade and retrograde movement of sperm from the epididymis [44]. As sperm are present in the ejaculate despite an absence of sexual activity, it must be assumed that tunica albuginea contraction occurs subconsciously and without the need for physical sexual stimulation. In this study, the rabbit tunica albuginea demonstrated marked spontaneous activity at a rate of approximately 140/h and the human tunica albuginea also demonstrated spontaneous contraction at a lower rate of 75 contractions/h. Spontaneous contractions of the human and rabbit testis have been previously reported [16, 23].

Concern has been raised over progressively falling sperm counts [1, 2]. It is possible that this is, in part, due to a failure of sperm transport rather than spermatogenesis. Developmental studies have demonstrated that the development of testicular smooth muscle is coincidental with sexual maturity [45–47]. This study has demonstrated an ultrastructural and pharmacological basis for testicular capsule contraction, which may play an essential role in moving sperm from the testis to the epididymis. This process is common to all species examined. Purinergic cotransmission was evident in the rabbit, and purinergic responsiveness was demonstrated in man, rats, and mice. We suggest that continuous flux of spermatids from the seminiferous tubules within the testis into the rete testis and epididymis may be caused in part by low-pressure, spontaneous contractions of the smooth muscle of the tunica albuginea. Larger volume flux may be caused by slow, sustained contractions induced by sympathetic, parasympathetic, and purinergic stimuli, as may be induced by sexual arousal. In man, this process is dominated by the adrenergic system in association with a smaller purinergic component, that may be consistent with purinergic cotransmission, but is without a parasympathetic component. Sympathetic stimulation may have a role in improving sperm counts.

ACKNOWLEDGMENTS

Mr. J. Bellringer and Mr. P. Thomas are thanked for their assistance in providing human tissue and Dr. Gillian E. Knight for editorial assistance.

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