Changing P2X receptor localization on maturing sperm in the epididymides of mice, hamsters, rats, and humans: a preliminary study

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Objective: To study using immunohistochemistry the localization of P2X receptor subtypes on the head of immature sperm in the human, mouse, hamster, and rat caput epididymidis.
Design: Basic research.
Setting: University-based hospital.
Patient(s): Three human epididymides were obtained from patients undergoing orchidectomy for metastatic prostate cancer.
Main Outcome Measure(s): $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_4$ receptor immunolocalization on sperm.
Result(s): In the present study, $P2X_{1,2,\text{ and }3}$ receptor localization was immunohistochemically demonstrated on the head of immature sperm in the human, mouse, hamster, and rat caput epididymidis. $P2X_4$ receptor immunostaining was also observed on the head of sperm in the caput epididymidis of mice, hamsters, and humans, but not rats. There was a subsequent loss of receptor staining on sperm in the cauda epididymidis, except in humans where staining of $P2X_4$ receptors persisted. Comparision with peanut agglutinin (PNA) binding studies suggested the P2X receptors were located on the acrosome membrane. $P2X_{5-7}$ receptors were examined but found to be absent.
Conclusion(s): The change in localization of receptor subtypes is coincidental with the functionally essential morphologic and maturational changes seen in sperm as they travel through the epididymis, and is suggestive of a role for purinergic signaling in sperm maturation and possibly fertility. (Fertil Steril® 2010;93:1415–20. ©2010 by American Society for Reproductive Medicine.)

Key Words: ATP, epididymis, PX receptors, sperm

The daily sperm production from the human testes is estimated to be between 45 and 207 million sperm per day (1). Sperm leaving the testis are immature, and lack both forward motility and zona pellucida binding capability (2). These functions are gained during the passage through the epididymis, following the interaction of sperm with the epididymal microenvironment and epididymal epithelium (3, 4). The epididymal epithelium progressively changes along the length of the epididymis, and the epididymal microenvironment is extremely complex, containing >100 proteins (5, 6). The epididymis is common to most male mammals, and is macroscopically divided into three regions—the caput (head), corpus (body), and cauda (tail)—with each section being microscopically distinct (4). The epididymis length is variable, being approximately 4 meters in humans (7) and approximately 80 meters in horses (8). The epididymal transit time mirrors this length variation, being between 2 and 16 days depending on species (1).

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The family of P2X receptors are ion-gated channels whose primary ligand is adenosine triphosphate (ATP). These receptors are increasingly being found to have novel trophic and apoptotic actions within tissues, besides their established role in purinergic cotransmission and pain initiation (9, 10). Sperm synthesize ATP, and although seminal fluid and sperm have a high ATP content, there is no correlation with fertility (11–13). Anatomically dependent P2 receptor localization has been noted on the mouse epididymal epithelium. Immunohistochemically, $P2X_{1,2,4,\text{ and }7}$ receptors have been demonstrated on clear cells in the corpus and proximal cauda epididymidis epithelium, with only $P2X_2$ receptors being localized on the clear cells of the initial segment. Reverse transcriptase polymerase chain reaction (RT-PCR) also demonstrated $P2X_4$ and $P2Y_1$ receptors in all epididymal segments as well as $P2Y_2$ receptor localization in the caput and corpus epididymidis epithelium. Dynamic function of these receptors was confirmed by the demonstration of calcium flux on stimulation with ATP, and it was suggested that these receptors are important for the ionic maintenance of epididymal fluid (14). Epididymal cell culture studies also support ATP-induced changes in the anion and fluid secretion by the epididymis (15). The complex membranous changes, termed the acrosome reaction, which sperm undergo before fertilization, can be induced by ATP and such stimulated sperm have higher fertilization rates (16). Previously, $P2X_{2\text{ and }3}$ receptor

immunostaining has been demonstrated on the acrosome of testicular rat spermatids in developmental stages I to VIII, and $P2X_5$ receptor localization in stages X to XIII (17). To date, there are no reports of P2X receptor localization on epididymal sperm, despite the demonstration of P2X receptors on more immature sperm within the testis, evidence for purinergic signaling within the epididymis, and improved fertility rates with ATP-activated sperm. This study investigated the localization of P2X receptors on maturing sperm within the epididymis of mice, hamsters, rats, and humans.

MATERIALS AND METHODS

Epididymides were obtained from adult Sprague-Dawley rats (weight: 345–360 g; n = 4), golden hamsters (weight: 240–265 g; n = 3), and mice (C57/BL10; weight: 32–37 g; n = 5) kept in accordance with Home Office (UK) regulations. All animals were housed in conditions of 12 hours light/12 hours dark, with access to food and water ad libitum. Rodents were killed in accordance with Schedule 1 (Animals Scientific Procedures Act, 1986) by a rising concentration of CO_2 and death was confirmed by cervical dislocation. Testes were examined before dissection and no abnormalities were observed. Human epididymides (n = 3) were obtained from patients undergoing orchidectomy for metastatic prostate cancer following informed consent. Institutional review board approval was obtained for the word performed and the use of human material. Epididymides were collected into Krebs solution, divided into the head and tail sections, and then embedded in Tissue Tek OCT compound (Sakura, Zoederwoude, the Netherlands), before being snap-frozen in isopentane and precooled in liquid nitrogen. The tissues were sectioned at 12 μm using a cryostat (Leica CM 3050, Nussloch, Switzerland), thaw mounted onto either gelatin-coated or polygalactin-coated slides, and air dried at room temperature. A section from the head (whole caput) and tail section of each epididymis was placed adjacently onto a single slide to ensure identical experimental conditions. The slides were stored at −20°C and allowed to return to room temperature for at least 10 minutes before use.

The avidin–biotin technique was used, as described by Llewellyn-Smith (18, 19) for P2X receptor immunohistochemistry. 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 minutes. To inactivate endogenous peroxidase, the sections were then treated with 50% methanol containing 0.4% hydrogen peroxide for 10 minutes. Nonspecific binding sites were blocked by incubating with 10% normal horse serum (NHS) in phosphate-buffered saline containing 0.05% thimerosal (Merthiolate) for 20 minutes. The $P2X_{1–7}$ receptor antibodies (Roche, Palo Alto, CA) were diluted to 0.25–5 μg/mL (determined by prior titration) with NHS and the sections were incubated with the 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 1hour. Sections underwent a further incubation with extravidin peroxidase (Sigma Chemical Co., Poole, UK) at 1:1,000 for 1 hour. For studies using peanut agglutinin (PNA) lectin, known to bind to the intact acrosome and therefore used to test for the integrity of the acrosome, the slides were fixed in 100% ethanol for 10 minutes and then incubated in FITC–PNA lectin (Sigma) diluted 10 μg/mL for 30 minutes at 37°C.

The reaction product was visualized using the nickel–DAB enhancement technique or using streptavidin FITC immunofluorescence. The specimens were dehydrated in xylene and mounted in Eukitt. Controls were performed with preimmune IgG and antibodies preadsorbed with the homologous peptides or omission of the primary antibody; minimal staining was observed under such conditions.

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.

RESULTS

$P2X_1$ receptors were localized by immunostaining on the sperm head from sperm contained in the caput epididymidis of mice (Fig. 1A), hamsters, rats and humans. This immunostaining was most pronounced in the most proximal sections of the caput epididymidis, and in the rat, staining was only present on sperm contained in the ducti efferenti. In the rodent species, staining reduced progressively through the epididymis and was absent on sperm in the cauda epididymidis. The presence of immunostaining was noted in sperm in the human cauda epididymidis. The smooth muscle surrounding the distal cauda epididymidis in the mouse strongly expressed $P2X_1$ receptors (Fig. 1B).

$P2X_2$ receptors were demonstrated in a similar pattern to $P2X_1$ receptors, with staining on the sperm head of sperm in the caput epididymidis, but staining was absent on sperm in the cauda epididymidis. Immunostaining was only seen on sperm in the most proximal sections of cauda epididymidis in humans and rats (Fig. 1C and D).

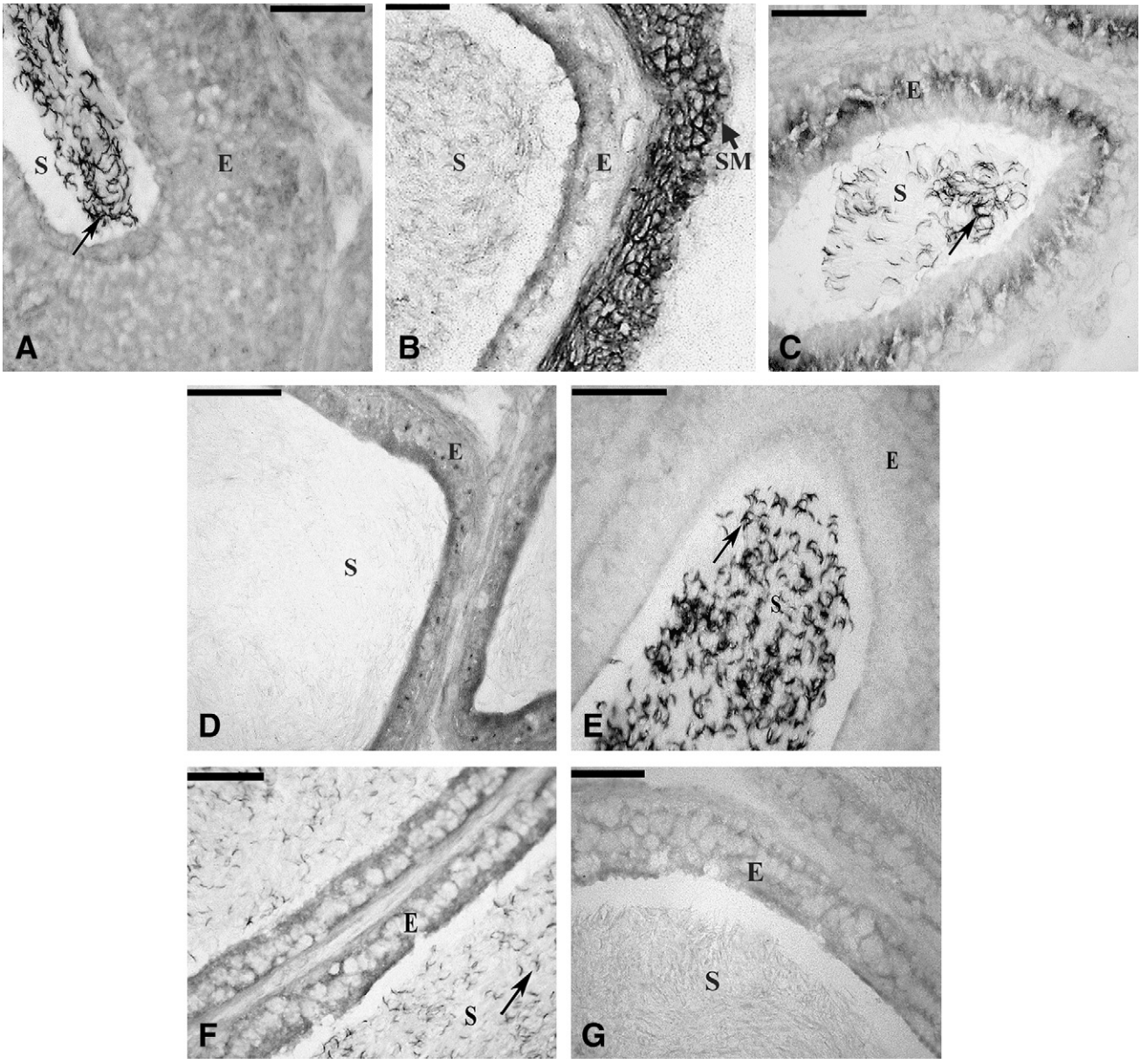
$P2X_3$ receptor immunostaining was demonstrated on sperm heads on the sperm of the caput epididymidis from all species, with subsequent loss of staining on sperm in the cauda epididymidis.

$P2X_4$ receptor immunostaining was observed on sperm from the caput epididymidis in all species except the rat. In the hamster and mouse, staining was strong in the caput epididymidis (Fig. 1E) and declined throughout the epididymis. Some staining persisted in the proximal cauda epididymidis (Fig. 1F) but was absent on sperm in the most distal cauda epididymidis (Fig. 1G). In humans, $P2X_4$ receptor staining was seen on caput epididymidis sperm (Fig. 2A) as well as on sperm contained in the most distal cauda epididymidis (Fig. 2B).

No immunostaining for $P2X_{5,6,\text{ or }7}$ receptors was observed on the sperm in either the caput or cauda epididymidis in any species examined.

FIGURE 1

P2X receptor-positive immunolocalization shown as black precipitate, indicated by thin arrows. S = sperm, E = epithelium, SM = smooth muscle. Scale bars = 100 μm. (**A**) Photomicrograph showing sperm in mouse caput epididymidis staining for $P2X_1$ receptors. (**B**) Photomicrograph showing sperm in the mouse cauda epididymidis no longer staining for $P2X_1$ receptors. However, there is strong staining of $P2X_1$ receptors on the smooth muscle (*thick arrow*) surrounding the distal tubule of the cauda epididymidis. (**C**) Photomicrograph showing sperm in the rat caput epididymidis staining for $P2X_2$ receptors. (**D**) Photomicrograph showing sperm in the rat cauda epididymidis no longer staining for $P2X_2$ receptors. (**E**) Photomicrograph showing sperm in the hamster caput epididymidis staining for $P2X_4$ receptors. (**F**) Photomicrograph showing a reduction in staining for $P2X_4$ receptors on sperm in hamster cauda epididymidis. (**G**) Photomicrograph showing a loss of staining for $P2X_4$ receptors on sperm in the most distal tubule of the hamster cauda epididymidis.

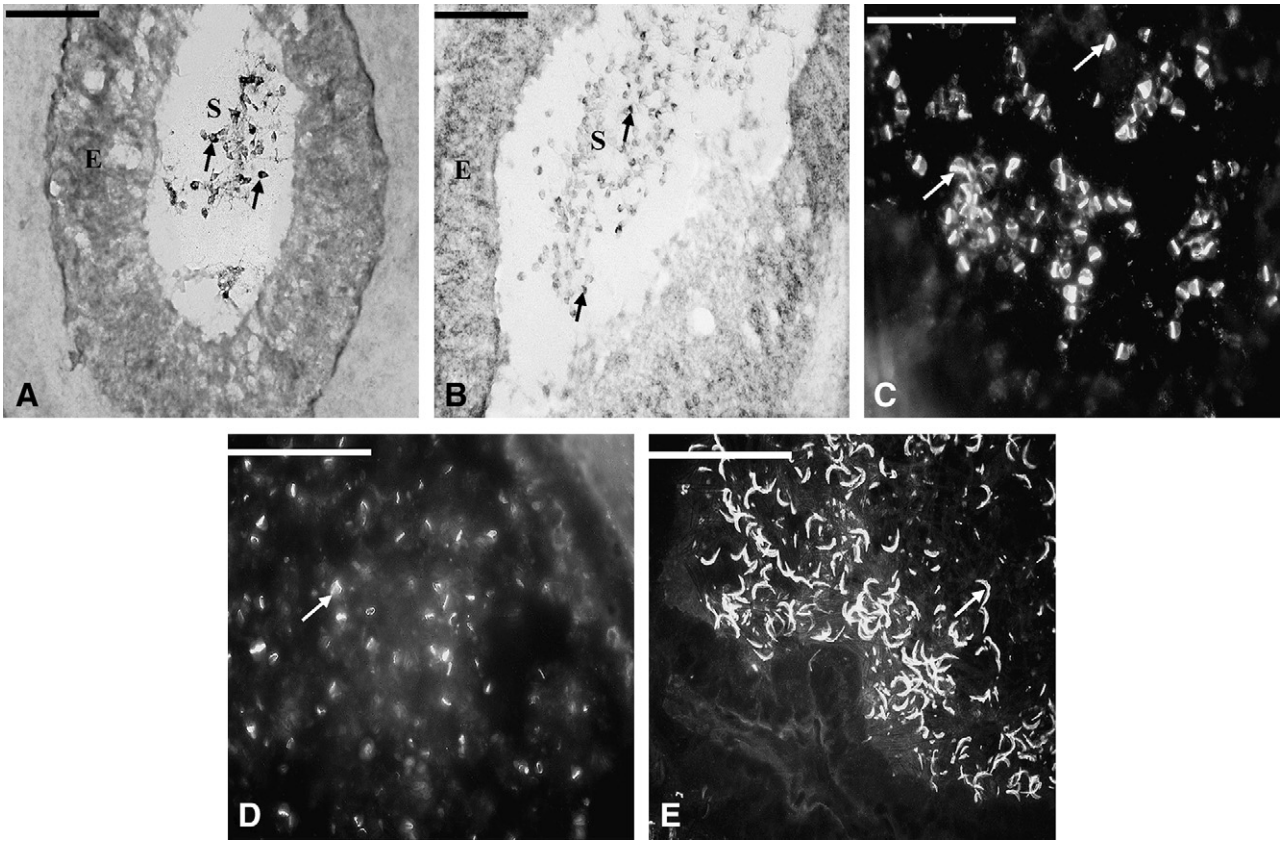


Banks. P2X receptors on maturing sperm. Fertil Steril 2010.

The density of human sperm within the epididymal lumen was markedly less than the other species examined, and many tubule cross sections did not demonstrate sperm. Significant debris was seen in sections of distal cauda epididymidis of rats and humans, consistent with apoptotic sperm. It was therefore important to ensure the integrity of the acrosome.

FIGURE 2

P2X receptor-positive immunolocalization, indicated by black precipitate or bright fluorescence. Examples of positive staining on sperm indicated by thin arrows. S = sperm, E = epithelium. Scale bars = 50 μm. (**A**) Photomicrograph showing sperm in human caput epididymidis staining for $P2X_4$ receptors. (**B**) Photomicrograph showing residual staining for $P2X_4$ receptors on sperm in the human cauda epididymidis. (**C**) Photomicrograph showing fluorescent PNA binding to the symmetrical acrosome of human sperm in caput epididymidis. (**D**) Photomicrograph showing fluorescent $P2X_1$ receptor staining on the symmetrical acrosome of human sperm in caput epididymidis. (**E**) Photomicrograph showing fluorescent PNA binding to the asymmetrical acrosome of rat sperm in caput epididymidis in a typical sickle pattern.



Banks. P2X receptors on maturing sperm. Fertil Steril 2010.

PNA lectin is known to bind to the intact acrosome (20), and in this study it confirmed the integrity of the acrosome on sperm in both caput and cauda epididymidis (Fig. 2C). In the symmetric acrosome of human and mouse sperm, PNA lectin predominantly bound to the equatorial segment of the acrosome. $P2X_1$ receptor immunostaining, when visualized using fluorescence, was found to be identical to that of PNA lectin (Fig. 2D). In the sickle-shaped asymmetric rat sperm the PNA staining bound to the acrosome in a classical sickle pattern (Fig. 2E).

DISCUSSION

This study has demonstrated, for the first time, that $P2X_{1,2 \text{ and } 3}$ receptors are localized on the head of immature sperm contained within the caput epididymidis of mice, rats, hamsters, and humans. There is a subsequent loss or reduction of $P2X_{1,2, \text{ and } 3}$ receptor immunostaining on mature sperm in the cauda epididymidis, although the presence of $P2X_4$ receptors on sperm in the cauda epididymidis of humans was observed. The localization is probably on the acrosome, as the immunohistochemical pattern was similar to that of PNA, which is known to bind to the intact acrosome. The developing rat spermatid, within the testis, has been shown to differentially express P2X receptors. $P2X_2$ and $P2X_3$ receptors were demonstrated together on developing spermatids in stages I to VIII on the developing acrosome, and $P2X_5$ receptors in stages X to XIII (17). The observed P2X receptor localization and subsequent loss, is coincidental to the key maturational stage of the epididymis. Anatomically, the initial segment of the epididymis has a distinct epithelium with a high epithelial cell height supportive of a nutrient or

absorptive function where immature sperm have $P2X_{1-4}$ receptor staining, which contrasts with the low epithelium seen in the cauda epididymidis, where the staining does not appear, which appears structurally more appropriate for storage of mature sperm (21). Estimates of epididymal sperm storage suggest that 52% of epididymal sperm are stored in the cauda, 23% in the corpus, and 25% in the caput, which appears consistent between species (4).

In the normal human epididymis 22.9% of sperm from the distal caput were motile in comparison to 68.3% from the mid to distal corpus with a slight reduction in the cauda (22). Human microcanulation studies suggest that the development of motility appears suddenly at the junction of the distal caput and proximal corpus epididymidis (23), seemingly where there is a progressive loss of P2X receptor staining. The ability of human epididymal sperm to bind to zona-free hamster oocytes increase with successive epididymal segments, and that only sperm from the cauda epididymidis are able to penetrate the oocyte (24). Human studies involving fertility rates following epididymovasostomy, suggest that only passage through the caput epididymidis is required for fertility, although fertility rates increased with more distal anastomoses (25).

During epididymal sperm maturation, the plasma membrane undergoes remodeling by the uptake of secreted glycoproteins, removal, and use of phospholipids from the inner leaflet of the bilayer, and processing of existent glycoproteins (3). Over 200 proteins have been identified in epididymal fluid, many are specific to different regions of the epididymis (26). The epithelial-sperm signaling and ionic changes that initiate and control these complex events are far from understood. The presence of $P2X_{1-4}$ receptors on immature sperm, but not mature sperm, may indicate a role for purinergic signaling in sperm maturation. It is also possible that the removal or loss of P2X receptors from sperm may be important, or indeed necessary, for the sperm to become fertile.

Ionic changes induced by stimulation of P2 receptors may be important in either maintaining or altering epididymal fluid composition to facilitate sperm membrane changes. This is supported by human studies on ejaculated sperm that have shown ATP rapidly induced the acrosome reaction, and that the mode of action was via the activation of Na^+ channels and was Ca^{2+}-independent. Functional in vitro fertilization studies using sperm from patients with male factor infertility, demonstrated a significantly higher fertilization rate, when the acrosome reaction was induced by exogenous ATP (16, 27, 28).

Seminal fluid has a high ATP content, generally viewed as an energy substrate (29), and although reduced seminal ATP levels are implicated in infertility (30), investigative studies have failed to find a correlation between seminal ATP and infertility (12, 31, 32). Sperm are known to have mitochondria concentrated around the midpiece of the spermatozoa and synthesize ATP; thus, seminal fluid ATP levels remain constant in the presence of substrates for ATP synthesis (11, 13), perhaps the source of ATP for the P2X receptors on the immature sperm head. Sperm also contain high intracellular levels of ATP (33, 34) and the concentration of ATP in the sample of seminal fluid is an important parameter for evaluating the fecundity of sperm. Apart from its role as the direct source of energy for sperm motility and fertilization (35), ATP is also suggested to be released from sperm during their transit through the male reproductive tract to regulate their own microenvironment (15, 36). Therefore, extracellular ATP may act as a neurotransmitter, as well as a paracrine/autocrine agent in the male reproductive system.

It has previously been proposed that epididymal spermatozoa can control their own fluid environment by releasing ATP into the lumen of epididymis to regulate the secretory activity of the epididymis (37), although another possible source of ATP may be from apoptotic sperm. It has also been claimed that ATP is released from epididymal cells (38) and ATP and adenosine are released from Sertoli cells as well as germinative and myoid peritubular cells of the seminiferous tubules (39). Previous studies have demonstrated the importance of innervation to the epididymal fluid composition (40). Neurogenic release may be a further source of ATP as it is known to be co-released with noradrenaline and acetylcholine in the stimulation of smooth muscle contraction of the genital tract (41, 42). In the present study there was strong $P2X_1$ receptor immunostaining of the smooth muscle surrounding the cauda epididymidis of the mouse, and it has been shown previously that $P2X_1$ receptors mediate contractions of the cauda epididymidis in rats (43). Absence of these receptors in $P2X_1$ receptor knockout mice has been shown to result in infertility (44) because of the decreased contractile activity in the vas deferens, but contractions of the cauda epididymidis may be even more important, given that it is the main storage site of sperm.

The loss of receptor localization in the distal epididymis was clearly evident, and may imply that the receptor is altered, possibly following stimulation, resulting in loss of localization, which may be part of a negative feedback loop. Alternatively, the localization may be masked by a proteinacous substance present in distal epididymal fluid. Several studies have demonstrated the presence of proteins specific to regions of the epididymis (45, 46), which may have an inhibitory role (47). The persistence of $P2X_4$ receptor staining in the distal cauda epididymidis suggests that masking is not universal or receptor alteration is more likely.

This study has demonstrated the localization of several P2X receptors on the heads of immature epididymal sperm in four different species, which shows a high degree of species similarity. This localization appears to be lost on more mature sperm in the distal epididymis, although this is incomplete for $P2X_4$ receptors in humans. This may suggest that ATP has an extracellular signaling role in the complex process of sperm maturation and possibly fertility.

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