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Expression of Nucleotide P2X Receptor Subtypes during Spermatogenesis in the Adult Rat Testis

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Key Words

P2X receptors · Testis · Seminiferous epithelium · Blood vessels · Immunohistochemistry · Rat

Abstract

The expression of ATP-gated ion channels (P2X receptors) was investigated in testes of adult rats by immunohistochemistry and Western blotting with antibodies against all seven P2X receptor subtypes. Immunoreactive cells were identified and monitored during germ cell maturation. Results of immunohistochemical and Western blotting experiments showed the expression of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_7$ receptors, while $P2X_4$ and $P2X_6$ receptors were absent from the testis. Blood vessels displayed immunostaining for $P2X_1$ and $P2X_2$ receptors; the $P2X_1$ receptors were present exclusively in blood vessels. $P2X_2$, $P2X_3$ and $P2X_5$ receptors were found to be expressed differentially in the various germ cell types throughout the different stages of the cycle of the seminiferous epithelium; $P2X_2$ and $P2X_3$ receptors were always observed together in the same cell types and at the same stages. Sertoli cells also showed differential staining for $P2X_2$ and $P2X_3$ receptors during the cycle of the seminiferous epithelium, whereas $P2X_7$ receptor expression was present throughout all stages. No immunostaining for P2X receptors was detected on Leydig cells. The possible roles of purinergic signalling in the control of germ cell maturation are discussed. In particular, it is suggested that purinergic signalling may play a role in controlling the maturation of germ cell subsets of different developmental ages that exist alongside each other in the adult testis.

Abbreviations used in this paper

DAB	diaminobenzidine
FSH	follicle-stimulating hormone
PAS	periodic acid-Schiff reaction
PBS	phosphate-buffered saline
RT	room temperature
SDS	sodium dodecyl sulfate

Introduction

The role of extracellular nucleotides as intercellular messenger molecules is nowadays well established [Burnstock, 1972, 1997]. The receptors for extracellular nucleotides are divided into two broad categories, the G protein-coupled P2Y receptors and the transmitter-gated cation channels named P2X receptors [Abbracchio and Burnstock, 1994]. Currently eight subtypes of mammalian

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P2Y receptors and seven subtypes of P2X receptors are known [Ralevic and Burnstock, 1998].

P2 receptors have been shown to play an important role in neurotransmission [Burnstock, 1996], in signalling in cellular growth and differentiation [Abbracchio, 1996] and in the mediation of apoptosis [Chow et al., 1997]. The potential role of P2 receptors has so far been investigated mainly by pharmacological studies and by molecular biology methods [North and Barnard, 1997]. In recent years, antibodies against each of the seven P2X receptor subtypes became available to us. In former studies, we demonstrated the specificity of these P2X receptor antibodies [Oglesby et al., 1999] and reported the differential cellular distribution of P2X receptors in differentiating and apoptotic squamous epithelia [Gröschel-Stewart et al., 1999a, b]. Among other immunohistochemical studies, we have shown the expression of P2X receptors in the genital tract male of rats [Lee et al., 2000].

In this current study we extend our immunohistochemical investigations of the male genital system with P2X receptor antibodies to the adult rat testis. This organ consists mainly of stratified epithelium and shows intense cellular proliferation, differentiation and apoptosis [Billig et al., 1995; Dym and Fawcett, 1970]. The testis provides the environment for spermatogenesis and it is the source of testosterone, needed for male bodily functions.

The testis consists of seminiferous tubules, within which spermatogenesis takes place, and interstitial spaces between these tubules, containing Leydig cells (testosterone-producing cells), as well as supporting tissue and blood or lymphatic vessels [Dym and Fawcett, 1970; Burkitt et al., 1993]. Germ cells and Sertoli cells are the only cell types present within the seminiferous tubules and they are in close contact with each other. The differentiating germ cells migrate within the seminiferous tubules and differentiate from stem spermatogonia, through spermatocytes, to spermatids. During this process they gain their haploid karyotype (after cycles of mitosis and meiosis), lose most of their cell volume and undergo fundamental structural transformation [Russell, 1977; Hess, 1990]. The changes in Sertoli cell and germ cell morphology during the repetitive cycle of germ cell development in the rat have been categorised into the '14 different developmental stages (I–XIV) of the cycle of the seminiferous epithelium' [Leblond and Clermont, 1952]. Germ cell development has been subdivided into '19 different steps (1–19) of spermatogenesis' [Leblond and Clermont, 1952; Hess, 1990].

In cell culture experiments, extracellular ATP and its analogues have previously been shown to have profound effects on testicular cells of the rat. P2 receptors were found to have a role in steroidogenesis in Leydig cells [Foresta et al., 1996b]. Rudge et al. [1995] have shown that Sertoli cells express $P2Y_2$ receptors. Purinergic agonists and antagonists altered the secretion of trophic factors from Sertoli cells [Meroni et al., 1998] and their hormone responsiveness [Filippini et al., 1994]. Foresta and coworkers have shown that extracellular ATP is a trigger of the acrosome reaction in human sperm [Foresta et al., 1992] and that the reaction is mediated by a P2X receptor [Foresta et al., 1996b].

In the present study we have investigated the expression of P2X receptors throughout the various developmental stages of the seminiferous epithelium in the testes of adult rats by immunohistochemistry and Western blotting.

Material and Methods

Animals

Breeding, maintenance and killing of the animals used in this study complied with all requirements of the national laws and regulations and were under control of the Home Office. Animals were kept at a constant 12/12 h light-dark cycle with water and food ad libitum. Rats were killed with increasing concentrations of carbon dioxide and death was confirmed by cervical dislocation. Tissues were taken from 3-month-old male Sprague-Dawley rats.

Immunohistochemistry

Testes from 4 rats were removed quickly and put immediately into ice-cold Hanks' balanced salt solution, pH 7.5 (Gibco BRL, UK). Tissues were embedded in Tissue-Tek (Sakura Finetek, Netherlands) without fixation and frozen in isopropanol precooled in liquid nitrogen. Cryostat sections (10 μm) were cut and placed on gelatine-coated slides.

Sections for immunohistochemistry for all P2X receptor antibodies were postfixed for 2 min at room temperature (RT) in 4% formaldehyde (BDH Laboratory Supply, UK) in 0.1 *M* phosphate buffer. Inactivation of endogenous peroxidase was carried out in 50% methanol and 0.3% H_2O_2 (30 min at RT).

Before incubation with the P2X antibodies nonspecific binding sites were blocked by preincubation with 10% normal horse serum (Harlan Sera-Lab, UK) in phosphate-buffered saline (PBS) for 20 min at RT, as previously described in detail [Glass et al., 2000]. An indirect immunohistochemical method with three layers of antibodies was used. Purinoceptor antibodies from rabbit were allowed to react with biotinylated donkey anti-rabbit antibody and were detected with avidin coupled horseradish-peroxidase and nickel-intensified diaminobenzidine (DAB) (giving a black colour precipitate) or with plain DAB (DAB without nickel, giving a brown colour precipitate). The P2X subtype-selective antibodies (obtained from Roche Bioscience, Palo Alto, Calif., USA) were raised in rabbits against a specific 15-amino-acid residue at the carboxy-terminus of each purinoceptor molecule [Oglesby et al., 1999]. The specificity of P2X antibodies was immunohistochemically determined by replace-

ment of primary antibody with nonimmune rabbit serum and by preabsorption with their cognate antigens.

Sections adjacent to those used for P2X antibody immunohistochemistry were counterstained with haematoxylin/periodic acid Schiff (PAS). These counterstained sections were assessed according to the criteria originally established by Leblond and Clermont [1952] and by the criteria reported by Hess [1990] in order to determine the stages of the cycle of the seminiferous epithelium in each region. The sections used for immunohistochemistry were counterstained with haematoxylin alone.

Images were examined using an Edge 200 light-microscope (Edge Scientific Instruments, Calif., USA) and a Nikon FDX 35 camera, and a Zeiss Axioplan microscope (Zeiss, Germany). Films were scanned with a Nikon LS-1000 scanner using the Adobe-Photoshop 5.0 program and an Apple Power-Macintosh G3. Prints were made with an Epson Stylus Photo 700 printer.

Western Blots

Testes were taken from two 3-month-old male Sprague-Dawley rats and were cut into halves. One half of each testis was immediately snap-frozen in liquid nitrogen for use in Western blotting, the other half was processed for immunostaining. Tissue for Western blotting was ground up under liquid nitrogen, using a mortar and pestle. The tissue powder was dissolved in 8 *M* urea (at 4°C), containing 2% sodium dodecyl sulphate (SDS). The homogenate was drawn through a 21-gauge needle to shear DNA, and was then cleared by centrifugation at 10,000 *g* for 30 min at 4°C. Western blotting was performed on the supernatant using a Mini-Protean 2 Electrophoresis and Trans-Blotting Cell (Bio-Rad, USA) according to the manufacturer's instructions.

Proteins were loaded on Tris-HCl Ready Gels (10% gel; Bio-Rad, USA) and run under reducing conditions (10% SDS and 26 m*M* dithiothreitol). Biotinylated molecular weight markers were obtained from Sigma (UK) and from Amersham (UK). Proteins were transferred onto a Hybond ECL-nitrocellulose membrane (Amersham, UK). Nitrocelluloses were then blocked (PBS containing 3% non-fat milk powder and 0.05% Tween 20) at RT and incubated in PBS, containing 3% non-fat milk powder and 2.5 µg/ml antibody. For detection of the immunoblots, the ECL chemiluminescence method was performed using a peroxidase-linked donkey anti-rabbit IgG, peroxidase-linked streptavidin and ECL Western Blotting Reagents (all from Amersham, UK). The signal was visualised on a Hyperfilm ECL (Amersham, UK) and scanned with a Umax Powerlook 2 flatbed-scanner, using software and computer as above.

Results

Western Blotting

Results of Western blot experiments using P2X receptor antibodies and crude testis extracts from adult rats are shown in figure 1. Under reducing conditions, antibodies for $P2X_1$, $P2X_3$, $P2X_5$ and $P2X_7$ detected two distinct protein bands of approximately 70 and 140 kD. After incubation with the $P2X_2$ antibody, four bands of approximately 30, 42, 70 and 140 kD were seen. Experiments using antibodies for $P2X_4$ and $P2X_6$ did not show any immunoreactivity.

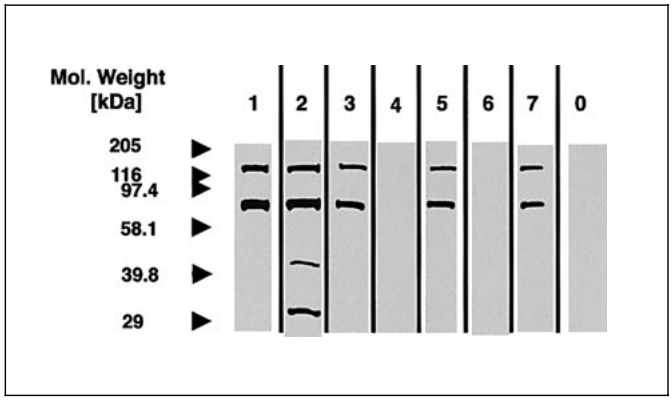


Fig. 1. Western blotting of crude extracts from adult rat testis. The Western blot shows results from different gels, run under reducing conditions. Molecular weights of marker proteins run with the lysates are given in kD, migration of the marker proteins is indicated by arrowheads. After incubation with $P2X_1$ receptor (lane 1), $P2X_3$ receptor (lane 3), $P2X_5$ receptor (lane 5) and $P2X_7$ receptor (lane 7), 2 bands of approximately 70 and 140 kD were detected; immunoreactivity for $P2X_2$ receptors (lane 2) was seen at four different bands of approximately 30, 42, 70 and 140 kD; no immunopositivity for $P2X_4$ receptors (lane 4) or for $P2X_6$ receptors was detected. Omission of the primary antibody (lane 0) led to absence of immunoreactivity.

P2X Receptors on Blood Vessels

Immunoreactivity for P2X receptors on blood vessels is shown in figure 2. Staining in vascular smooth muscle was seen after incubation with antibody for $P2X_1$ receptors, which could be completely preabsorbed with its cognate peptide. Immunostaining for $P2X_2$ receptors was also detectable in vascular smooth muscle. Immunostaining for P2X receptors in vascular smooth muscle was not altered by the stage of the cycle of the adjacent seminiferous epithelium.

P2X Receptors in Seminiferous Tubules

An overview of the distribution in the adult rat testis of cells immunoreactive to $P2X_2$, $P2X_3$, $P2X_5$ or $P2X_7$ antibodies is given in figure 3. No staining for $P2X_1$ receptors was observed in the seminiferous tubules and immunoreactivity was not seen on any tissue sample with antibodies for $P2X_4$ or $P2X_6$ receptors.

Immunostaining for $P2X_2$ receptors was detected in cells close to the basement membrane of the seminiferous tubules, in cells of the central region of the seminiferous epithelium and in the adluminal area of the tubules during stages I to VIII of the cycle of the seminiferous epithelium (fig. 3A–C). Infrequently, myoid cells surrounding the seminiferous tubules were immunostained for $P2X_2$.

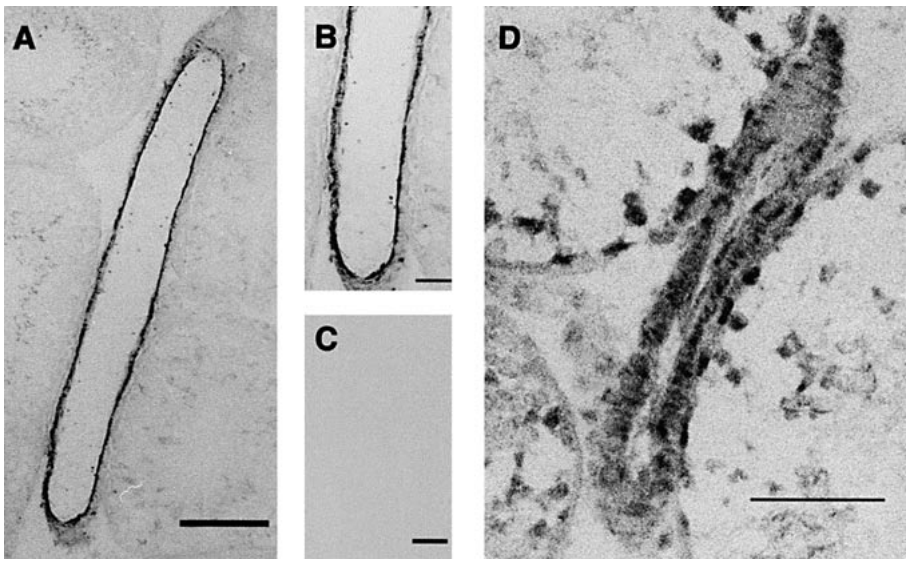


Fig. 2. Immunoreactivity for P2X receptors on blood vessels of adult rat testes. Fresh-frozen testes of adult rats were processed for the immunodetection of P2X receptors and staining for P2X receptors was developed with the DAB/nickel method. **A** Blood vessel immunoreactive for $P2X_1$ receptors. **B** A magnified part of the blood vessel stained for $P2X_1$ in **A**. **C** An area corresponding to the vessel in **B** on an adjacent section was preabsorbed for $P2X_1$ antibody. **D** Immunostaining for $P2X_2$ receptors in a blood vessel, adjacent to two seminiferous tubules, which also show $P2X_2$ receptor immunostaining. Bars: **A** = 150 μm; **B**, **C** = 50 μm; **D** = 40 μm.

Staining for $P2X_3$ receptors was also detected in areas close to the basement membrane and in central and adluminal parts of the seminiferous tubule during stages I to VIII. Spermatids at stages VI, VII and VIII could clearly be seen at lower magnification (fig. 3E).

Incubation with antibody to $P2X_5$ receptors showed staining of germ cells in stages IX to XIII (fig. 3H), being most intense at stage XII, and also gave staining in some myoid cells surrounding the tubules.

At lower magnification, staining for $P2X_7$ receptors was only detected in the peripheral area of the seminiferous tubules and was present throughout all stages of the cycle of the seminiferous epithelium (fig. 3J). Higher magnification of $P2X_7$-immunopositive cells revealed their location close to the basement membrane of the tubules by normal light microscopy (fig. 3K) and by phase contrast microscopy (fig. 3L).

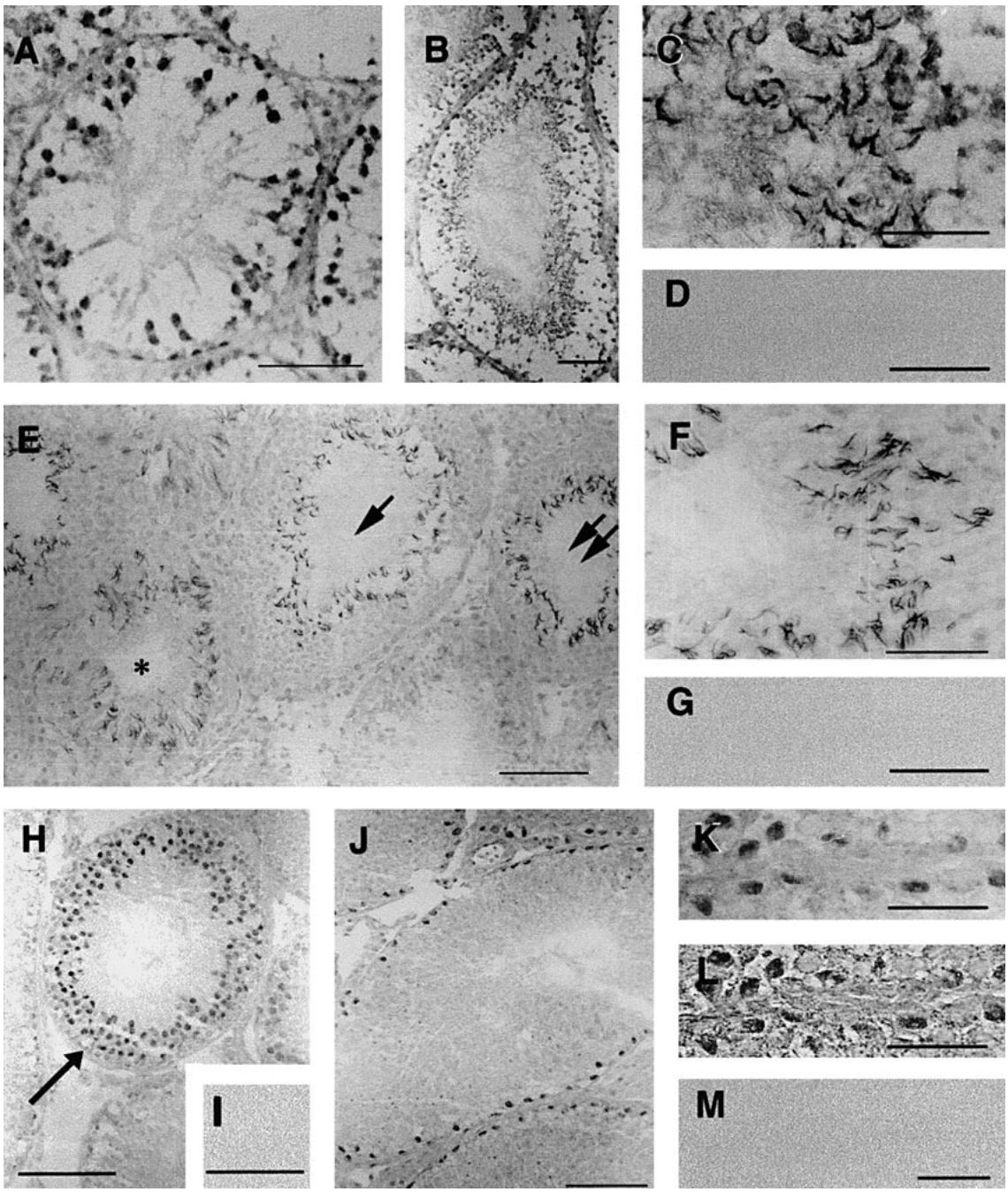
Preabsorptions for $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_7$ receptors immunolabelling seminiferous tubules were performed successfully (fig. 3D, G, I, M).

Identification of P2X-Immunopositive Cells

At higher magnification, cells showing immunostaining for P2X receptors (with the plain DAB method) could be identified by counterstaining with haematoxylin (fig. 4). The results of identification of P2X-immunopositive cells in the seminiferous epithelium are summarised in table 1.

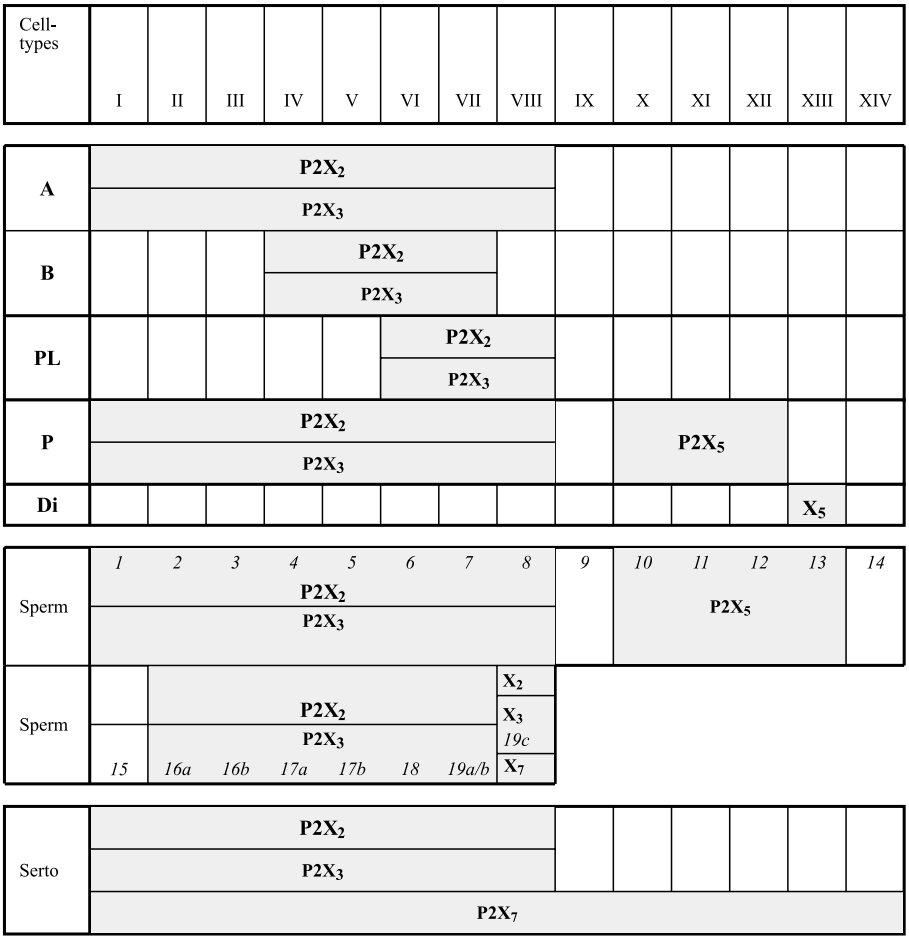
Immunoreactivity for $P2X_2$ receptors was found in the Sertoli cell perinuclear region throughout stage I to stage VIII of the cycle, being weaker at stages I, II, VI, VII and VIII, and more intense at stages III, IV and V. Staining for $P2X_2$ receptors could be seen at various stages in developing gametes. $P2X_2$ receptor immunoreactivity appeared on the cell membranes of type A spermatogonia and pachytene spermatocytes throughout stages I to VIII. Type B spermatogonia were labelled through stages IV to VI and preleptotene spermatocytes were stained for $P2X_2$ receptors through stages VI to VIII. In step 2 through to step 8, spermatids staining for $P2X_2$ receptors appeared to be in the area of the developing acrosome, seeming most intense at step 3. Late step 16 and early and late step 17 spermatids (stages III to V of the cycle of the seminiferous epithelium) were intensely labelled for $P2X_2$ receptors. Immunolabelling for $P2X_2$ was most abundant when the spermatids moved close to Sertoli cell nuclei and sometimes continuous labelling was detected between the Sertoli cell perinuclear region and groups of late step 16 and step 17 spermatids. Intense staining for $P2X_2$ was also seen on the convex site of step 18 to late step 19 spermatids (fig. 4A, B).

Fig. 3. Overview of P2X receptors in seminiferous tubules of adult rat testes. The distribution of immunoreactivity for P2X receptors using the DAB/nickel technique is shown. **A** Immunoreactivity for $P2X_2$ receptors in a seminiferous tubule at stage III of the cycle. Cells in the peripheral area of the tubule and round and elongated structures in the central parts of the seminiferous epithelium are stained. **B** Immunoreactivity for $P2X_2$ receptors at stage VII, showing immunostaining of cells in the basal compartment and in the adluminal region. **C** The sickle-shaped structures in the luminal part of the



tubule in **B** at higher magnification. **D** Staining is absent after a preabsorption experiment for $P2X_2$ receptors in a seminiferous tubule at stage VII. **E** Immunostaining for $P2X_3$ receptors in sickle-shaped luminal structures, and in elongated structures located in the adluminal compartment. Staining for $P2X_3$ receptors is shown in a tubule at stage IV (asterisk), in a tubule at stage VI (double arrows), and in a tubule at stage VII (arrow). **F** A magnified area of the adluminal part of the tubule at stage VI in **E**. **G** Staining for $P2X_3$ receptors was completely abolished after preabsorption of the antibody (shown in adluminal area of a tubule at stage VI). **H** Immunoreactivity for $P2X_5$ receptors in cells located adluminally within the seminiferous epithelium in a tubule at stage X (arrow). Surrounding seminiferous tubules at stages earlier than stage IX are immunonegative. **I** Immunostaining for $P2X_5$ receptors is completely abolished after preabsorption in a seminiferous tubule at stage X. **J** The distribution of immunoreactivity for $P2X_7$ receptors in various adjacent tubules is restricted to the outer border of the tubules. **K, L** Immunostaining for $P2X_7$ receptors close to the walls of two adjacent seminiferous tubules. **K** Normal light microscopy. **L** Phase contrast microscopy. **M** Absence of immunostaining for $P2X_7$ receptors after preabsorption on a consecutive slide in an area corresponding to **L**. Bars: **A, B, E, H–J** = 80 μm; **C, D, F, G, K–M** = 30 μm.

Table 1. Summary of P2X-immunopositive cells in the seminiferous tubules throughout the l4 stages of the seminiferous epithelium

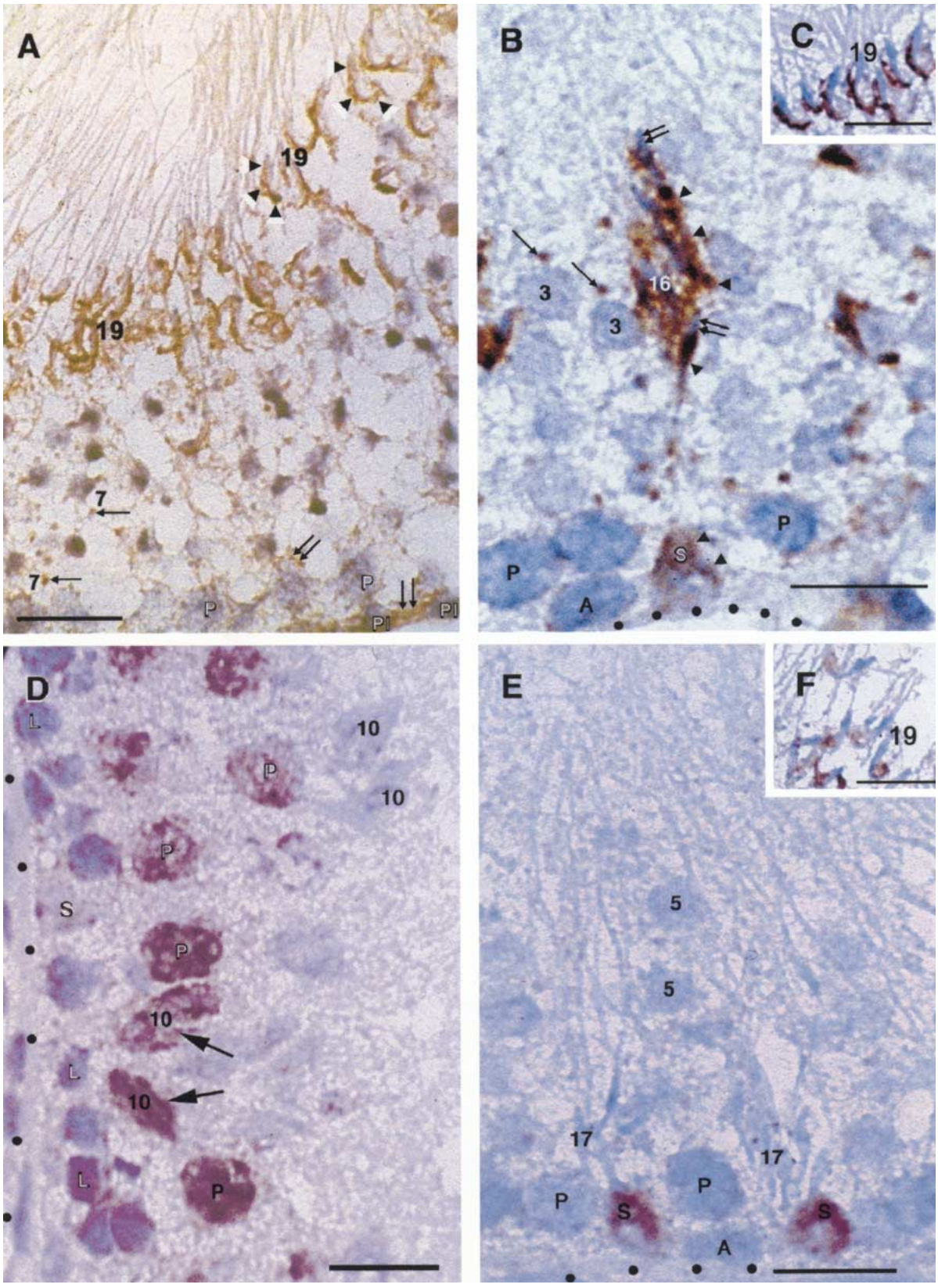


The stages of the cycle of the seminiferous epithelium are given in Roman numerals.

Shaded boxes indicate the presence of immunopositive cells for a single P2X receptor subtype throughout the respective stages of the cycle. Italic numerals indicate the developmental steps of spermatid maturation. Throughout stages I to VIII younger (1–8) and older (16a–19c) generations of spermatids coexist, whereas through stages IX to XIV only one generation of developing spermatids is present.

X_2 = $P2X_2$ receptor; X_3 = $P2X_3$ receptor; X_5 = $P2X_5$ receptor; X_7 = $P2X_7$ receptor; A = type A spermatogonia; B = type B spermatogonia; P = pachytene spermatocytes; Di = diplotene spermatocytes; PL = preleptotene spermatocytes; Sperm = spermatids; Serto = Sertoli cells.

Staining for $P2X_3$ receptors closely resembled the findings described above for $P2X_2$ receptors. Sertoli cell perinuclear regions were labelled for $P2X_3$ receptors throughout stages I to VIII of the cycle of the seminiferous epithelium, the labelling being most abundant at stages III, IV and V. Type A spermatogonia and pachytene spermatocytes were immunoreactive for $P2X_3$ receptors through stage I to VIII. Type B spermatogonia were labelled for $P2X_3$ receptors from stage IV to stage VI and preleptotene spermatocytes were stained for $P2X_3$ from stage VI through stage VIII. The developing acrosoma of step 2 to step 8 spermatids labelled for $P2X_3$ receptors, the staining being most abundant at step 3. Throughout stages III to V of the cycle of the seminiferous epithelium late step 16, early step 17 and late step 17 spermatids were intensely immunopositive for $P2X_3$ (fig. 4B). The most intense staining for $P2X_3$ receptors was found in spermatids of step 18, and early and late step 19 at the convex site of the spermatids (fig. 4C).



(For legend see page 384)

Staining for $P2X_5$ receptors was seen in pachytene spermatocytes through stages X to XII of the cycle of the seminiferous epithelium, and in the diplotene spermatocytes of stage XIII. Immunoreactivity for $P2X_5$ receptors on spermatids was detected from stages X to XIII, being most intense at stage XII. At stage X, spermatids (step 10) of the more adluminal parts of the seminiferous tubule were immunonegative for $P2X_5$, but became progressively more stained for $P2X_5$ as they moved towards the perinuclear region of the Sertoli cells (fig. 4D). Labelling for $P2X_5$ was seen in pachytene spermatocytes, and in step 10 spermatids close to the periphery of the seminiferous tubule, but the adluminal step 10 spermatids were unstained (fig. 4D).

Fig. 4. (page 383). Identification of P2X receptor immunopositive cells in testes of adult rats. The identity of P2X receptor immunoreactive cells could be revealed using higher magnification DAB method for colour precipitation and light counterstaining with haematoxylin. **A** Immunostaining for $P2X_2$ receptors in a section of a seminiferous tubule at stage VII. The preleptotene spermatocytes (Pl) in the peripheral area of the tubules are strongly immunopositive for $P2X_2$ receptors; pachytene spermatocytes (P) are more weakly labelled (double arrows); round shaped step 7 spermatids (7) in the central part of the seminiferous epithelium label for $P2X_2$ receptors in the area of their developing acrosome (arrows); the elongated step 19 spermatids (19) in the luminal part of the tubule are stained at their convex side. **B** Immunostaining for $P2X_3$ receptors in a section at stage III. The approximate location of the basement membrane is indicated by a dotted line; the perinuclear region of a Sertoli cell (S) is labelled for $P2X_3$ receptors (arrowheads); type A spermatogonium (A) and some pachytene spermatocytes (P) are labelled on their cytoplasmic membranes; the area of the developing acrosome in (round shaped) step 3 spermatids (3) is stained for $P2X_3$ receptors (arrows); elongated step 16 spermatids (16, double arrows) are labelling for $P2X_3$ receptors as they move closer to the perinuclear region of a Sertoli cell (arrowheads). **C** Immunolabelling for $P2X_3$ receptors at the convex side of elongated, luminal step 19 spermatids (19). **D** Incubation with antibody for $P2X_5$ receptors in a section at stage X; the dotted line indicates approximate location of the basement membrane; Sertoli cells (S) and leptotene spermatocytes (L) are unlabelled for $P2X_5$ receptors, whereas pachytene spermatocytes (P) are strongly stained; elongating step 10 spermatids (10) in the luminal part of the tubule are unstained, but step 10 spermatids that already moved closer to the perinuclear region of Sertoli cells become immunolabelled (arrows). **E** Immunostaining for $P2X_7$ receptors in a section at stage V; the dotted line outlines the approximate location of the basement membrane; several cell types unstained for $P2X_7$ receptors are visible: type A spermatogonia (A), pachytene spermatocytes (P), round step 5 spermatids (5) and elongated step 17 spermatids (17) are all unlabelled. Sertoli cells (S) show staining for $P2X_7$ receptors in their perinuclear region. **F** Immunostaining for $P2X_7$ receptors at the concave side of step 19 spermatids (19). Bars = 20 μm.

Immunoreactivity for $P2X_7$ receptors was seen in Sertoli cells throughout all stages of the cycle of the seminiferous epithelium. Figure 4E shows two Sertoli cells immunolabelled for $P2X_7$ receptors at stage V. Other cell types like type A spermatogonia, step 5 spermatids and step 17 spermatids were not immunostained. The only other structure displaying staining for $P2X_7$ is the concave site of late step 19 spermatids (fig. 4F).

Discussion

The specificity of our antibodies against P2X receptors has previously been proven on Western blots using lysates from cells transfected with P2X receptors [Oglesby et al., 1999]. In the current study we examined the specificity of our antibodies for P2X receptors by Western blotting of crude extracts from testes of adult rats. The molecular weights for $P2X_1$, $P2X_3$, $P2X_5$ and $P2X_7$ receptors were estimated as approximately 70 and 140 kD. These findings resemble the results obtained by immunoblotting for P2X receptors of crude extracts of adult rat thymus [Glass et al., 2000]. Immunoblotting for $P2X_2$ receptors gave a different result, displaying two additional bands of approximately 30 and 42 kD. A molecular weight band for $P2X_2$ receptors in the range of 40 kD has been reported from immunoblots of crude extracts from rat cardiac and smooth muscle [Worthington et al., 1999]. In the current study there was an absence of immunoreactivity for $P2X_4$ receptors from the adult rat testis in histochemical and immunoblotting experiments. However, in a previous study from our group it was shown by Northern blotting that mRNA for $P2X_4$ receptors is expressed in rat testis [Bo et al., 1995], with the abundance of a second, smaller band next to the 1.9-kB band obtained for full-size $P2X_4$ mRNA. A smaller-sized $P2X_4$ mRNA may point to the occurrence of a splice variant of the $P2X_4$ receptor in the rat testis. Splice variants for $P2X_4$ receptors have repeatedly been found [Lê et al., 1997; Townsend-Nicholson et al., 1999]. A splice variant lacking the 15 amino acids of the carboxy terminus of the receptor (the antigenic sequence of our antibodies) would not be detected by the antibodies used in this study. Tanaka et al. [1996] reported the expression of $P2X_4$ mRNA by in situ hybridisation. This group used a mixture of probes against three different epitopes of $P2X_4$ mRNA, leaving it open which specific epitope of the receptor they detected.

Immunoreactivity for both $P2X_1$ and $P2X_2$ receptors was present in vascular smooth muscle of the adult rat testis. This is consistent with previous studies, showing

the expression of $P2X_2$ as well as $P2X_1$ receptors in vascular smooth muscle of several vessels [Collo et al., 1996; Nori et al., 1998]. The autonomic innervation in the testes of most mammals has been shown to be composed mainly of excitatory noradrenergic fibres, supplying predominantly the blood vessels [Bell, 1972]. ATP has been shown to be a cotransmitter in noradrenergic nerves and to participate in the control of blood flow [Burnstock, 1990].

$P2X_2$ and $P2X_3$ receptors were both expressed in the same cell types and at the same stages of the cycle of the seminiferous epithelium and they were both present in the same steps of spermatid development. It is well established that $P2X_2$ and $P2X_3$ receptors are coexpressed in sensory neurones [Cook et al., 1997; Vulchanova et al., 1997] and can form heteromultimeric ion channels [Lewis et al., 1995]. Our immunohistochemical data suggest that heteromultimeric $P2X_{2/3}$ receptors may also be present in testicular cells.

In the present study differential staining for P2X receptor subtypes during the testicular maturation of germ cells is shown. The changes in receptor expression occurred homogeneously within a group of germ cells of the same developmental stage. The abundance of certain P2X receptors on one homogeneous group of cells, and the absence of P2X receptors on a different group of cells would separate these two groups with respect to their purinergic signalling. Since several developmental stages of germ cells are known to occur parallel to each other in the seminiferous epithelium [Leblond and Clermont, 1952], a specific mechanism to control and co-ordinate different groups of germ cells in their development may be indicated. A cell-type- and maturation stage-specific receptor expression, as shown for P2X receptors in this study, could account for coordinated purinergic signalling within one homogeneous group, separating it (in respect of purinergic signalling) from a different group not expressing P2X receptors.

Lalevée et al. [1999] speculated upon a different pathway for ATP release, experimenting with immature rat Sertoli cell cultures. These authors describe a possible ATP release from Sertoli cells after stimulation with follicle-stimulating hormone (FSH), and release of ATP by germ cells. Studies in cultured rat Sertoli cells [Filippini et al., 1994; Meroni et al., 1998] have shown that extracellular ATP has profound effects on FSH responsiveness of the Sertoli cells. Using the murine Sertoli cell-line TM4, Filippini et al. [1994] and Rudge et al. [1995] showed that purinergic signalling can be mediated by the $P2Y_2$ receptor (P2U receptor in these articles). Foresta et al. [1995] found that P2X receptors may also participate in the purinergic signalling of rat Sertoli cells. Taking together the evidence from these previous articles with the findings of this report, one might speculate that purinergic signalling can be mediated through P2X and P2Y receptors. Purinergic signalling may act in concert with hormones (like FSH) that are well known to be of importance in spermatogenesis. It seems unlikely that neuronal release of ATP is involved in control of sperm development since the testicular sympathetic innervation in most mammals is restricted to blood vessels and the capsule [Bell, 1972]. Carvalho et al. [1993] could not find a role for sympathetic innervation in the control of sperm development since there are no changes after chemical sympathectomy of adult rats.

$P2X_2$ and $P2X_3$ receptors were strongly expressed in developing germ cells until stage VIII of the cycle. Profound changes take place at stage VIII, as the mature generation of sperm is released [Leblond and Clermont, 1952] and Sertoli cells change their hormone responsiveness at this stage, especially their responsiveness to FSH [Parvinen et al., 1980]. One might speculate that this change in FSH responsiveness of Sertoli cells could account for changes in the magnitude of ATP secretion from Sertoli cells, according to the mechanism suggested by Lalevée et al. [1999]. In the subsequent stages (IX–XIV) $P2X_2$ and $P2X_3$ receptors were absent from the tubules but $P2X_5$ receptors were detected through stages X–XIII. At stage X of the cycle of the seminiferous epithelium step 10 spermatids start to elongate. During the process of spermatid elongation, the spermatids are engulfed by apical invaginations of Sertoli cells [Beach and Vogl, 1999]. At stage X and in the following stages these crypts deepen, bringing the spermatids closer to the perinuclear region of the Sertoli cells. Step 10 spermatids which were situated in the apical compartments of the Sertoli cells did not label for P2X receptors, whereas step 10 spermatids in the basal region of the epithelium were immunopositive for $P2X_5$ receptors.

We did not detect any staining for P2X receptors on the interstitial Leydig cells. This is in contrast to the pharmacological findings of Foresta et al. [1996b], who reported $P2X_7$ receptors (P2Z receptors in their article) on cultured rat Leydig cells. However, when we have immunostained cultured rat Leydig cells for P2X receptors, we detected a broad expression of P2X receptor subtypes [Glass, unpubl. observations]. Thus, there seem to be some differences in P2X receptor expression in vivo and in vitro.

$P2X_2$ and $P2X_3$ receptors were found in the area of the developing acrosome of spermatids. It has been shown

that during acrosome formation the majority of proteins synthesized in the endoplasmic reticulum is transported via the Golgi network to the acrosome located in the perinuclear region [reviewed by Toshimori, 1998]. The immunopositive, perinuclear area probably consists of the endoplasmic reticulum, the Golgi network and the acrosome, since the stained structure remains lobular in shape, whereas the acrosome itself flattens during development [Toshimori, 1998]. The acrosome reaction of human spermatozoa can be triggered via ATP-gated ion channels [Foresta et al., 1996a], and incubation of human sperm with ATP increases in vitro fertilization rates [Rossato et al., 1999].

Staining for $P2X_2$ and $P2X_3$ receptors was also found on the *convex* site of spermatids that are released at step 19. This staining occurs in a region where 'tubulobulbar complexes' have their origin. These tubulobulbar complexes are thought to be important for sperm-anchoring before release [Russell and Clermont, 1976] and in cell volume reduction of spermatids [Russell, 1979].

On the concave site of step 19 spermatids we observed staining for $P2X_7$ receptors. The greatest decrease in spermatid cell volume (about 70%) occurs between step 18 and step 19 spermatids [Russell, 1979]. This is thought to be achieved mainly through the movement of spermatid cytoplasm through tubulobulbar complexes into the Sertoli cell cytoplasm. At stages VII and VIII of the cycle the apical parts of Sertoli cells surround the step 19 spermatids and retain them in the adluminal region of the tubule. The transport of cytoplasm from mature spermatids into the Sertoli cells takes place at the concave site of the step 19 [Russell, 1979]. $P2X_7$ receptors can form membrane pores allowing the passage of relatively large molecules – up to 900 daltons [Steinberg and Silverstein, 1987; Surprenant et al., 1996]. Thus, it could be speculated that $P2X_7$ receptors form pores between step 19 spermatids and Sertoli cells through which the aqueous component of the spermatid cytoplasm could be removed.

In conclusion, we have shown differential, stage-dependent immunostaining for P2X receptors in testes of mature rats. Our results suggest that purinergic signalling via P2X receptors may take part in the control of co-ordinated germ cell maturation. P2X receptors could also participate in the mediation of cell volume reduction and spermatid release. However, functional studies will be needed to specifically address the role of P2X receptors in the testis.

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