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REGULAR ARTICLE

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P2 receptors in the thymus: expression of P2X and P2Y receptors in adult rats, an immunohistochemical and in situ hybridisation study

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Abstract The expression of the seven P2X receptor subtypes and of two P2Y receptors was examined immunohistochemically and by in situ hybridisation in thymi of adult male rats. $P2X_4$, $P2Y_{2\ and\ 4}$ receptor mRNA colocalisation studies combining in situ hybridisation and immunohistochemistry were also carried out. P2X and P2Y receptors were found on thymocytes. P2X receptors were also abundant in cells of the thymic microenvironment, involved in control of T-cell maturation in vivo. We are the first to describe the expression of $P2X_4$ receptors on thymocytes and confirm the finding of $P2X_1$ and $P2Y_2$ receptors on subpopulations of lymphocytes. $P2X_{1,2,3,4\ and\ 5}$ receptors were present in blood vessels of the thymus. $P2X_{1,2\ and\ 4}$ receptors were detected in vascular smooth muscle, while $P2X_3$ receptors appeared to be associated with endothelial cells; some small arteries were positive for $P2X_5$, possibly labelling vascular smooth muscle or fibroblasts in the adventitia. $P2X_{2,3,6\ and\ 7}$ receptors were found on thymic epithelial cells. $P2X_{2\ and\ 3}$ receptors were abundant on medullary epithelial cells, whilst $P2X_6$ receptors were prominent in Hassall's corpuscles. $P2X_2$ receptors were found on subcapsular and perivascular epithelial cells. $P2X_{2,6\ and\ 7}$ receptors were detected in epithelial cells along the thymic septa. Expression of P2X receptors was also investigated by Western blotting of crude thymic tissue extracts under reducing conditions. All seven P2X receptor subtypes were found to be dimers of approximately 70 kDa and 140 kDa molecular weight. ATP-mediated apoptosis and cell proliferation of thymocytes are discussed.

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Introduction

ATP has long been known as an intracellular energy carrier, but is also recognised as an extracellular signalling molecule (see Burnstock 1997). The role of ATP as a motor-neurotransmitter was first proposed by Burnstock in 1972, and many articles on its effects in the cardiovascular system, vascular and visceral smooth muscle, endothelial cells, secretory and endocrine cells and several types of blood cells are available today (Burnstock 1995).

Two families of receptors to purines and pyrimidines have been defined, the G-protein-coupled P2Y receptors and the ligand-gated cation channels named P2X receptors (Abbracchio and Burnstock 1994). Investigations in the field have been enhanced in recent years by the cloning and characterisation of P2X and P2Y receptor subtypes (Burnstock and King 1996). There are now seven subtypes of P2X receptors and five subtypes of mammalian P2Y receptors.

P2X receptors have been reported on blood and immune cells. The bifunctional $P2X_7$ subtype is a membrane pore for molecules up to 900 Da, in addition to its action as a fast and selective channel for small mono- and divalent cations (Surprenant et al. 1996). The $P2X_7$ receptor (formerly termed P2Z) has mainly been detected in mast cells and macrophages and has there been related to apoptosis and necrosis (Di Virgilio et al. 1989). It has also been suggested that it participates in mitogenic stimulation of thymocytes (which are intrathymic T-cells or T-lymphocytes), cytokine release from macrophages, formation of macrophage polykaryons and cytotoxicity (Collo et al. 1997). Another line of investigation of P2X receptors in the immune system has been raised since significant sequence homology of $P2X_1$ with the RP-2 gene, expressed in apoptotic thymocytes, was de-

scribed (Valera et al. 1994). In contrast, Koshiba et al. (1997) found transient upregulation of $P2Y_2$ but not $P2X_1$ in mouse thymocytes after inducing apoptosis in cultured T cells. $P2X_1$ receptor involvement in apoptosis is currently under intensive discussion (Chow et al. 1997). A good model for the study of immune cell development is the thymus, which is the major site of T-cell proliferation and maturation. In the thymus, T-lymphocytes gain self-compatibility and their antigen receptor repertoire, but also up to 95% of T cells can undergo apoptosis (Ritter and Crispe 1992). Antibodies are available to mark cells in distinct developmental phases and functions. Of fundamental importance to lymphocyte development is the thymic microenvironment of epithelial cells, interdigitating cells, macrophages, mast cells and fibroblasts (Ritter and Crispe 1992). The thymus may play an important role in neuroimmune interactions and gets extensive feedback from various other endocrine organs, also effecting T-cell development (Weihe et al. 1991).

The physiological role of P2 receptors may be discernible from their expression in well-defined subsets of these cells. The aim of the present study, therefore, was to investigate the expression of P2X and P2Y receptors in the whole range of thymic cells with immunohistochemical and in situ hybridisation methods. Western blotting was carried out to examine the specificity of antibodies and to investigate the molecular weight of native P2X receptors.

Materials and methods

Animals

Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with the UK national laws and regulations. Tissues were taken from 3-month-old male Sprague-Dawley rats (n=4). Animals were kept at a constant 12 h/12 h light-dark cycle with food ad libitum. Rats were killed by exposure to an increasing dose of carbon dioxide and death was confirmed by cervical dislocation.

Immunohistochemistry and immunofluorescence

Tissue handling

Thymi were removed quickly and immediately put in ice-cold Hanks' balanced salts solution, pH 7.5 (GIBCO BRL, Scotland). Unfixed tissues were embedded in Tissue-Tek (Sakura Finetek, Netherlands) and frozen in isopropanol precooled in liquid nitrogen. Cryostat sections (10 µm) were cut and placed on poly-L-lysine-coated slides. Tissues for immunohistochemistry were postfixed for 2 min at room temperature in 4% formaldehyde (BDH Laboratory Supply, UK) and 0.03% picric acid in phosphate-buffered saline (PBS**)**, or without picric acid for immunofluorescence studies. Inactivation of endogenous peroxidase was carried out in 50% methanol and 0.3% H_2O_2 (30 min) for immunohistochemistry. Tissues for immunofluorescence underwent a second fixation step in ice-cold (–20°C) acetone for 7 min. Blocking of nonspecific binding sites was achieved by preincubation with normal horse serum (NHS) (Harlan Sera-Lab, UK) at room temperature for 20 min, as described in detail by Llewellyn-Smith et al. (1993).

Immunostaining

An indirect immunohistochemical and immunofluorescent method with three layers of antibodies was used. P2X receptor antibodies from rabbit were allowed to react with biotinylated donkey anti-rabbit antibody and detected with avidin-coupled horseradish-peroxidase/nickel-intensified 3,3'-diaminobenzidine (DAB) or avidin-coupled Texas red. The P2X antibodies were obtained from Roche Bioscience (Palo Alto, CA). The P2X subtype-selective antibodies were each raised in rabbits against a specific 15 amino acid residue at the carboxy-terminus of each P2X receptor molecule (Oglesby et al. 1999).

Mouse antibodies were detected with a second layer of goat anti-mouse immunoglobulin G (IgG; Sigma, MA) and immunoreactivities were shown with a fluorescein isothiocyanate (FITC)-labelled chicken anti-goat antibody (ICN, Biomed, CA).

All primary antibodies were diluted in 10% NHS in PBS containing 0.05% thimerosal (TPBS) (Sigma), secondary antibodies in 1% NHS in TPBS, and tertiary antibodies in TPBS only. A mixture of monoclonal mouse antibodies against cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19 (anti-pan cytokeratin antibody from Sigma) was used to identify epithelial cells in colocalisation studies (Kendall 1991) and a monoclonal antibody for CD5 (clone OX19, Biogenesis, UK) was used to identify thymocytes (Barclay 1981). Controls included omission of all primary antibodies, and the specificity of P2X antibodies was further determined by preabsorption with their corresponding antigens. To determine possible cross-linking between rabbit and mouse antisera, single- and double-staining experiments with each antibody were made.

Cells were identified by their characteristic shape, localisation within the tissue and colocalisation with specific markers (Kendall 1991; Ritter and Crispe 1992). Counterstaining of P2X antibodies with contrast green (Kierkegaard and Perry, USA) was performed to distinguish histologically between cortical and medullary regions.

In situ hybridisation

Tissue handling was carried out as described above, with sections of 7 µm being postfixed for 10 min.

Probes

Antisense oligonucleotide probes 45 nucleotides in length for rat $P2Y_2$, rat $P2Y_4$ and rat $P2X_4$ were obtained from either Genosys (UK) or MWG Biotech (Germany) and were labelled at their 3'-end with the DIG oligonucleotide tailing kit (Roche Diagnostics)

Table 1 Antisense oligonucleotides from rat $P2X_4$, $P2Y_2$ and $P2Y_4$ comprising 45 nucleotides corresponding to the last 15 amino acids at the C-terminus of the protein

Oligonucleotide sequences	
Rat $P2X_4$ antisense oligonucleotide:	5'-CTGGTTCATCTCCCCCGAAAGACCCTGCTCGTAGTCTTCCACATA-3'
Rat $P2Y_2$ antisense oligonucleotide:	5'-GATGGCGTTGAGGGTGTGGCAACTGAGGTCAAGTGATCGGAAGGA-3'
Rat $P2Y_4$ antisense oligonucleotide:	5'-GACAATGTTCAGCACATGACAGTCAGCTTGCAACAGTCTTGCCTG-3'

according to the manufacturer's instructions. The sequences for the probes are shown in Table 1. The specificity of each probe was confirmed by screening the Genbank database. The $P2X_4$ probe corresponds to the nucleotides encoding the 15 amino acid sequence against which the $P2X_4$ antibody was raised.

Hybridisation

Tissues were dehydrated in ethanol (70%, 80%, 90% and 100%), air dried and incubated in pre-hybridisation buffer [50% formamide; ×2 saline sodium citrate buffer (SSC); ×1 Dennhardt's; 1 mg/ml denatured, sheared salmon sperm DNA (Sigma); and 1 mg/ml tRNA type X from bakers' yeast (Sigma)] for 1 h at 37°C in a humidified chamber. This was followed by incubation in hybridisation buffer (1 ng labelled probe in 1 µl prehybridisation buffer) at 37°C overnight. Washing of unhybridised probe was done as follows: 2×5 min in 2×SSC at room temperature, 2×15 min in 2×SSC at 37°C, 2×15 min in 1×SSC at 37°C, with a final stringency wash of 2×30 min in 0.5×SSC at 37°C. DIG-labelled probes were detected by anti-DIG-alkaline phosphatase labelled Fab fragments (Boehringer/Mannheim) as described elsewhere (Komminoth 1992). Negative controls included omitting of the probe and competing labelled probe with a 75-fold excess of unlabelled probe.

Tyramide amplification

The signal amplification procedure was used for visualisation of $P2X_3$ receptors and in colocalisation studies of P2 receptor protein and mRNA expression. In situ hybridisation was performed as described above. Then antibodies were applied as for immunohistochemistry (see above), and tyramide signal amplification (Renaissance, TSA indirect, NEN, USA) was performed according to the manufacturer's instructions. For visualisation a horseradish peroxidase/DAB stain was used.

Photography

Images were made with an Edge 200 light microscope (Edge Scientific Instruments, CA) and a Nikon FDX 35 camera, a Zeiss Axioplan microscope (Zeiss, Germany), and with an Edge H-160 fluorescence microscope and a Canon EOS Elan 2 camera. Fluorescence filters were purchased from Chroma Technology Group (VT, USA) allowing discrimination of FITC (470/540 nm) and Texas red labelling (546/610-nm excitation and emission bands, respectively). Double staining was shown with a triple-band bass filter set for DAPI/FITC/Texas red. Films were scanned with a Nikon LS-1000 scanner using the Adobe Photoshop 4.0 program and an Apple Power Macintosh G3. Prints were made with an Epson Stylus Photo 700 printer.

Western blots

Tissues were taken from two 3-month-old male Sprague-Dawley rats, carefully dissected from surrounding connective tissue and lymph nodes and immediately snap frozen in liquid nitrogen. The thymi were homogenised under liquid nitrogen, using a mortar and pestle. The tissue homogenates underwent three freeze/thaw cycles and were dissolved in 8 M urea, containing 2% sodium dodecyl sulphate (SDS).

These homogenates were used for Western blotting in 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) and run under reducing conditions (2% SDS and 26 mM dithiothreitol). Results of these experiments were compared with those obtained under conditions in which the protein was not only reduced but SH groups were also alkylated with iodoacetamide (Westermaier 1990). Biotinylated molecular weight markers were obtained from Sigma (B2787) and from Amersham (UK) (RPN 2107). Proteins were transferred onto a Hybond ECL-nitrocellulose membrane (Amersham). The nitrocellulose was then blocked (PBS containing 3% milk powder and 0.05% Tween 20) at room temperature and incubated overnight in the same solution containing 2.5 µg/ml antibody at 4°C. For detection the ECL chemiluminescence method was performed using a peroxidase-linked donkey anti-rabbit IgG, peroxidase-linked streptavidin and ECL Western blotting reagents (all purchased from Amersham). The signal was visualised on a Hyperfilm ECL (Amersham) and scanned with a Umax Powerlook 2 flatbed scanner, using software and computer as above.

Results

Western blots and preabsorption controls

Clear bands were obtained in Western blots under reducing conditions of crude thymic tissue extracts, where strong signals for each P2X receptor subunit were detected (Fig. 1).

Experimental conditions using iodoacetamide to alkylate SH groups produced identical results. For all P2X receptor subtypes clear bands of approximately 70 kDa and approximately 140 kDa were found (Fig. 1, lanes 1–7), while controls of the blots where primary or secondary antibodies were omitted resulted in complete absence of any staining (Fig. 1, lane 0; omitting primary antibody).

Specificity of the P2X antibodies was also shown by discrete immunohistochemical staining in different areas of the tissue and by all controls. Replacement of P2X antibodies with TPBS or non-immune rabbit IgG resulted in complete loss of staining. Results of the preabsorption experiments are shown below.

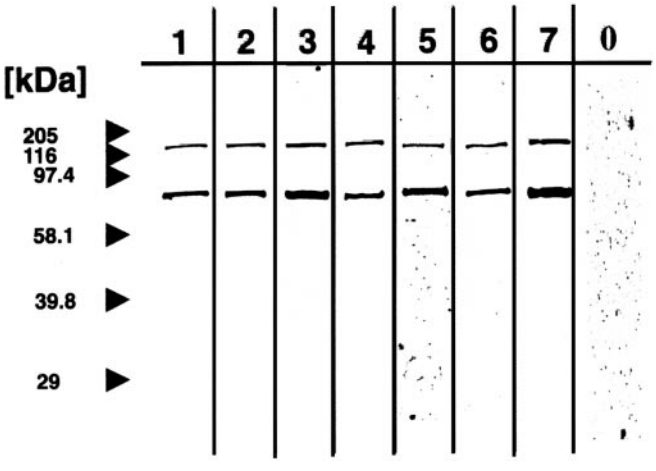


Fig. 1 Western blots of crude thymic tissue extracts from the rat. Western blots were carried out under reducing conditions in 10% polyacrylamide gels (4% stacking gel); separated proteins were transferred on ECL-nitrocellulose membranes and visualised by chemiluminescence. Thymi were taken from 3-month-old male rats. The panel shows Western blots after separation of proteins on seven different gels: molecular weight markers given in kilodaltons on the left (*lane 1* incubation with $P2X_1$ antibody, *2* $P2X_2$ antibody, *3* $P2X_3$ antibody, *4* $P2X_4$ antibody, *5* $P2X_5$ antibody, *6* $P2X_6$ antibody, *7* $P2X_7$ antibody, *0* omission of primary antibody)

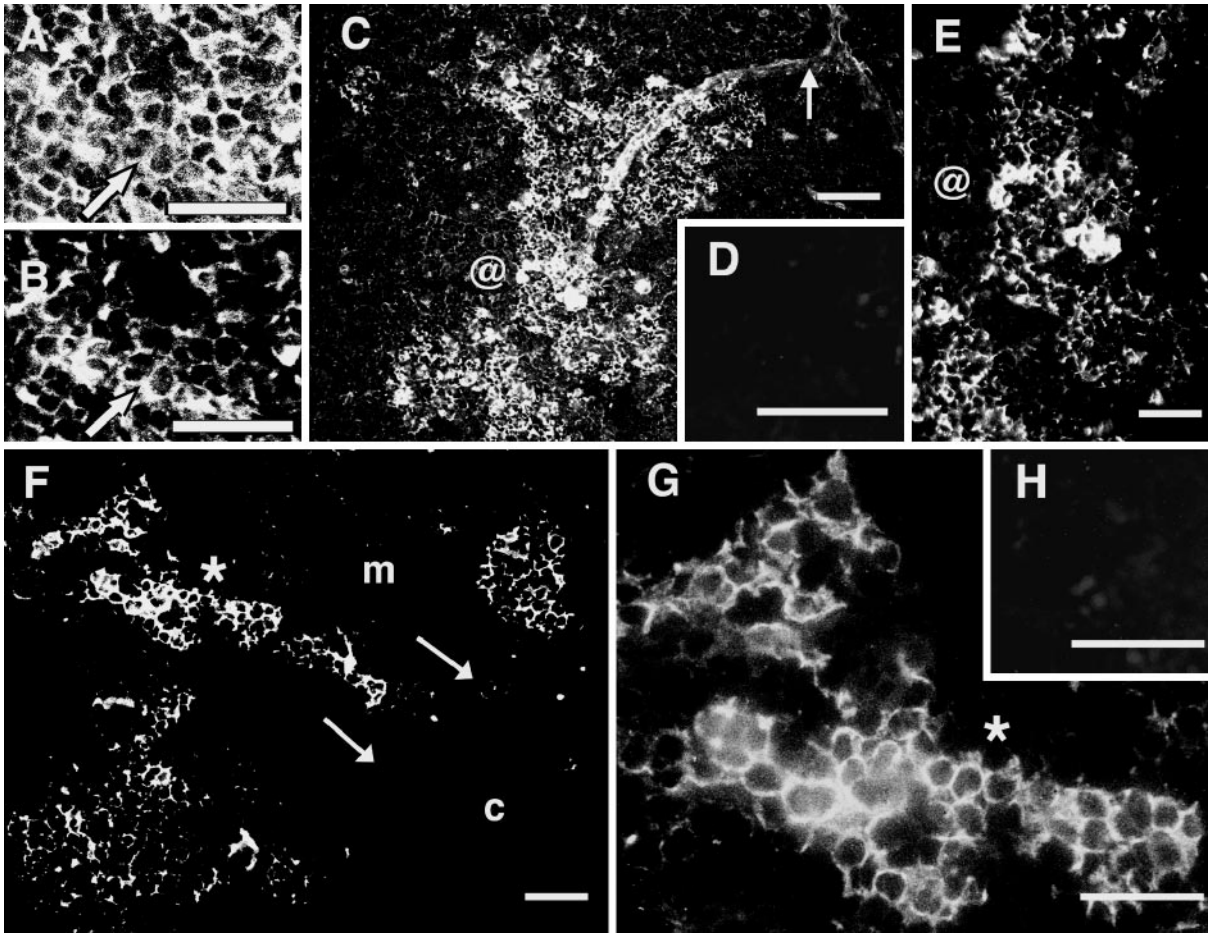


Fig. 2A–H $P2X_1$ and $P2X_4$ receptor immunoreactivity in rat thymocytes: immunofluorescence with Texas red. **A** Immunolabelling with an antibody against CD5 (OX-19) shows that the pan T-cell marker is colocalised with $P2X_1$-antibody staining (*arrows* in **A** and **B**). **B** Thymocytes staining for $P2X_1$ in the cortical area. **C** $P2X_1$-positive cells in the cortex also showing a stained septum (*arrow*). **D** Preabsorbed $P2X_1$ in an area corresponding to **C**. **E** Magnified part of **C** (marked as @). **F** Several clusters of $P2X_4$-expressing thymocytes along the corticomedullary junction and within the medulla (*m* medulla, *c* cortex, *arrows* indicate the corticomedullary boundary). **G** A magnified part of **F** (marked by *asterisk*). **H** Preabsorption of $P2X_4$ showing an area corresponding to **G**. *Scale bars* 40 μm **(A–F)**, 20 μm **(G,H)**

P2X receptors on thymocytes

Immunoreactivities for $P2X_1$ and $P2X_4$ receptors on thymocytes were seen using an immunofluorescence detection method (Fig. 2). The staining was compared with the thymocyte marker OX-19. Incubation with OX-19 gave an almost uniform staining of thymocytes (Fig. 2A). Immunoreactivities for $P2X_1$ receptors were observed solely in the thymic cortex (Fig. 2B,C,E). Immunopositive cells appeared in clusters and were colocalised with the thymocyte marker OX-19 (compared in Fig. 2A and Fig. 2B). Larger clusters of $P2X_1$-positive thymocytes were observed along the thymic septa (Fig. 2C). The immunoreactivity for $P2X_1$ in the septum may have originated from vascular smooth muscle of vessels that grow into the thymus within these septa (compare with Fig. 3A). Preabsorption of $P2X_1$ receptor antibody with its cognate peptide resulted in complete loss of staining. Immunoreactivity for $P2X_4$ receptors was found on thymocytes (identified by colocalisation with the thymocyte marker OX-19) in the thymic cortex and the medulla. $P2X_4$ receptors were again detected in larger clusters of thymocytes (Fig. 2F,G). These thymocyte conglomerates, sometimes reaching from the cortex deep into the medulla, contained densely packed cells, intensely stained for $P2X_4$. Single, scattered $P2X_4$-positive cells were also seen, but were not as intensely stained as the clusters of thymocytes (Fig. 2G). Preabsorption of $P2X_4$ receptor antibody with its corresponding peptide completely eliminated the immunostaining (Fig. 2H).

P2X receptors on blood vessels

Immunoreactivities for P2X receptors were abundant on blood vessels (Fig. 3). The $P2X_1$ antibody stained for vascular smooth muscle (Fig. 3A) of small and big arteries in the thymic septa and in the corticomedullary junction and could be preabsorbed (Fig. 3B). Immunoreactivity for $P2X_2$ receptors was mainly observed in the vascular smooth muscle of bigger blood vessels. $P2X_2$-posi-

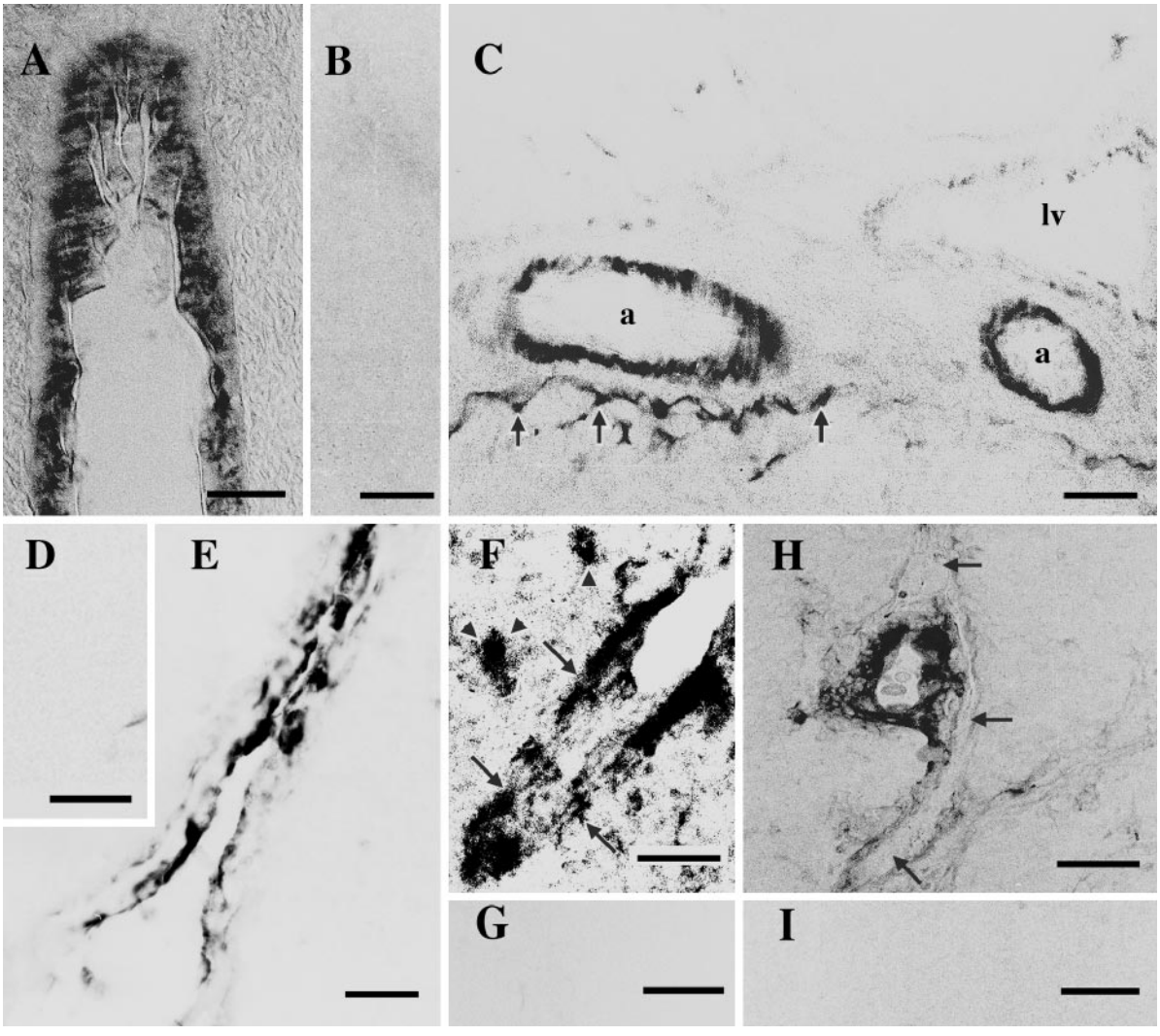
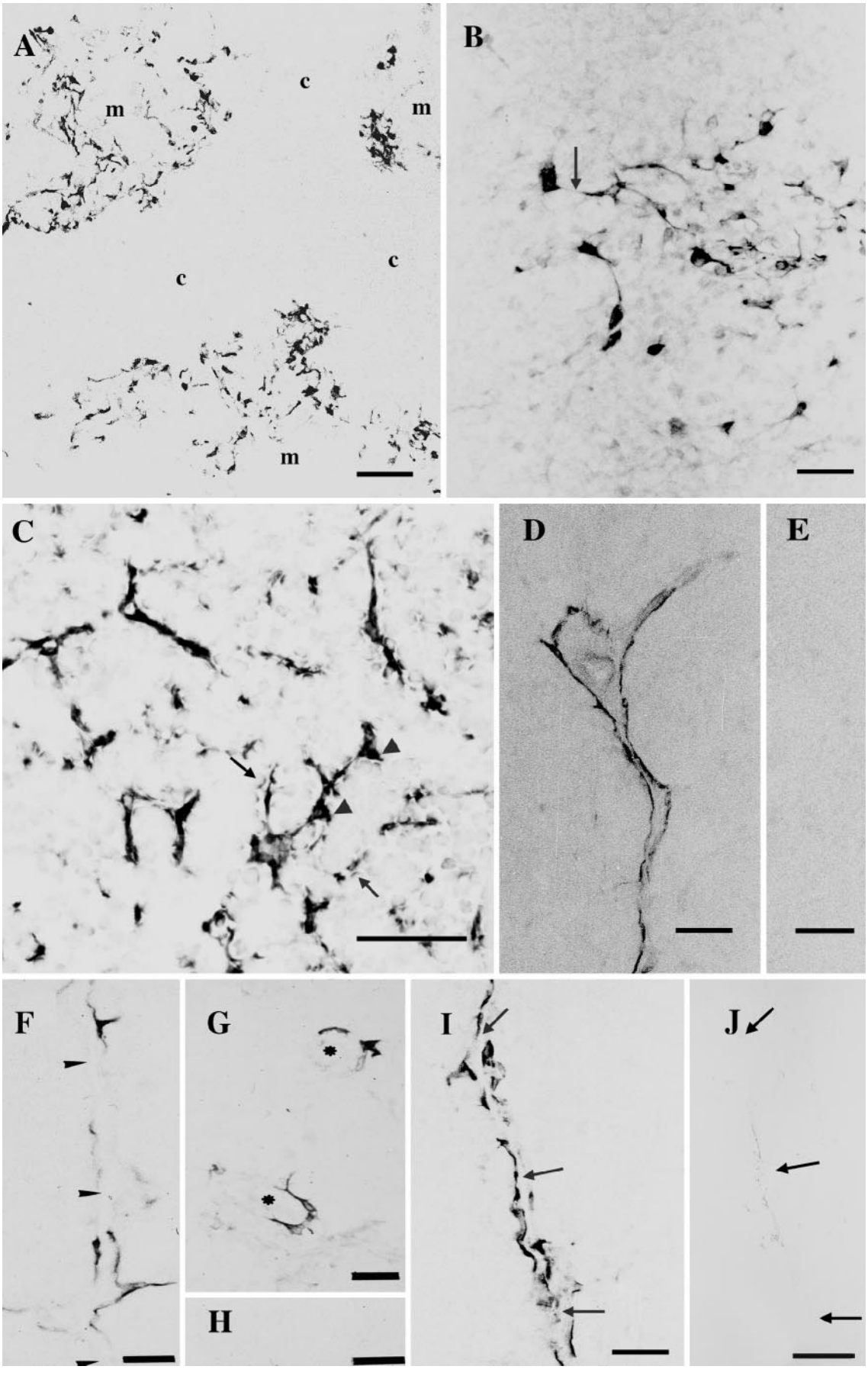


Fig. 3A–I Immunoreactivities for P2X receptors on blood vessels in rat thymus. Immunohistological results were obtained with the use of the nickel-intensified DAB/peroxidase method and with the use of tyramide amplification (**D,E**). **A** Immunoreactivity for $P2X_1$ receptors in vascular smooth muscle of a cortical artery. **B** Preabsorption of $P2X_1$ antibody in an area corresponding to **A**. **C** $P2X_2$-positive vascular smooth muscle in a vessel of the thymic septum surrounded by $P2X_2$-positive epithelial cells (*arrows*); the vascular smooth muscle of septal arteries (*a*) stains for $P2X_2$ receptors, whereas a nearby lymphatic vessel (*lv*) is immunonegative. **D** Preabsorbed $P2X_3$ antibody on an area corresponding to **E**. **E** $P2X_3$-positive structures in a septal vessel. **F** Immunoreactivity for $P2X_4$ receptors in a cortical vessel (*arrows*), surrounded by $P2X_4$-positive clusters of thymocytes (*arrowheads*). **G** Preabsorption of $P2X_4$ antibody in an area corresponding to **F**. **H** A septal vessel showing immunoreactivity for $P2X_5$ (*arrows* indicate the septum). **I** Preabsorption of $P2X_5$ antibody in an area corresponding to **H**. *Scale bars* 60 µm **(A–C**,**F–I)**, 80 µm **(D,E)**

tive vascular smooth muscle was predominantly seen in arteries of the thymic septa (Fig. 3C), whereas nearby lymphatic vessels were negative for $P2X_2$ receptor immunoreactivity. In fewer cases $P2X_2$ receptor-positive vascular smooth muscle cells were observed in large intralobular vessels, surrounded by connective tissue. In some smaller vessels of the thymus, immunoreactivity for $P2X_3$ receptors was observed after enhancement with biotinylated tyramide. The staining pattern for $P2X_3$ receptors appeared to be associated with endothelial cells (Fig. 3E) and could be preabsorbed under identical methodology (Fig. 3D). $P2X_4$-receptor-immunoreactive cells could be seen in the vascular smooth muscle of small thymic blood vessels in some septa and predominantly in cortical and medullary areas (Fig. 3F) that was also preabsorbable (Fig. 3G). Some immunostaining for $P2X_5$ receptors could be detected in thymic vessels. This was probably localised to vascular smooth muscle or to fibroblasts surrounding the vessel as the central cells of the vessel were immunonegative (Fig. 3H). The immunostaining disappeared after incubation of $P2X_5$-receptor antibody with its corresponding peptide (Fig. 5I).

P2X receptors on thymic epithelial cells

Immunoreactivity for P2X receptors was abundant on thymic epithelial cells (Fig. 4). Medullary epithelial cells (Fig. 4A–C) and epithelial cells of the thymic septa (Fig.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4D,F,G,I) were found to be immunopositive for P2X receptors. Occasionally perivascular (Fig. 3C) and subcapsular $P2X_2$-positive epithelial cells were detected. Medullary epithelial cells were regularly seen with strong immunoreactivity for $P2X_2$ receptors (Fig. 4A,B). Cell to cell contacts of $P2X_2$-positive medullary epithelial cells were visible (Fig. 4B) and formed a three-dimensional network, as found by staining of transversal and sagittal sections. After incubation with $P2X_3$ antibody immunoreactivity was again detected in medullary epithelial cells. $P2X_3$-positive medullary epithelial cells could be shown in close contact with surrounding thymocytes (Fig. 4C). $P2X_2$-positive thymic epithelial cells were regularly present on the thymic septa (Figs. 3C, 4D) and they were undetectable after preabsorption (Fig. 4E). $P2X_6$-receptor-immunoreactive thymic epithelial cells were less frequently observed. Antibody for $P2X_6$ receptors detected some epithelial cells in the septa (Fig. 4F) and marked epithelial cells forming the outermost layer of Hassall's corpuscles (Fig. 4G). The staining for $P2X_6$ receptors was completely preabsorbable (Fig. 2H). Immunoreactivity for $P2X_7$ receptors was detected in epithelial cells of some thymic septa (Fig. 4I) and this staining for $P2X_7$ receptors was at least greatly reduced after carrying out preabsorption of the antibody (Fig. 4J). No labelling for $P2X_7$ receptors could be seen in other cells of the thymus.

In situ hybridisation and double labelling

Double-labelling studies were carried out to identify subsets of epithelial cells and to show colocalisation of in situ hybridisation and immunostaining (Fig. 5). Identification of the epithelial-shaped cells immunoreactive for P2X receptors was carried out in colocalisation experiments with a mouse monoclonal anti pan-cytokeratin antibody mixture. Colocalisation experiments revealed that all P2X-positive reticular-shaped cells were epithelial cells, as shown in Fig. 5A for $P2X_2$.

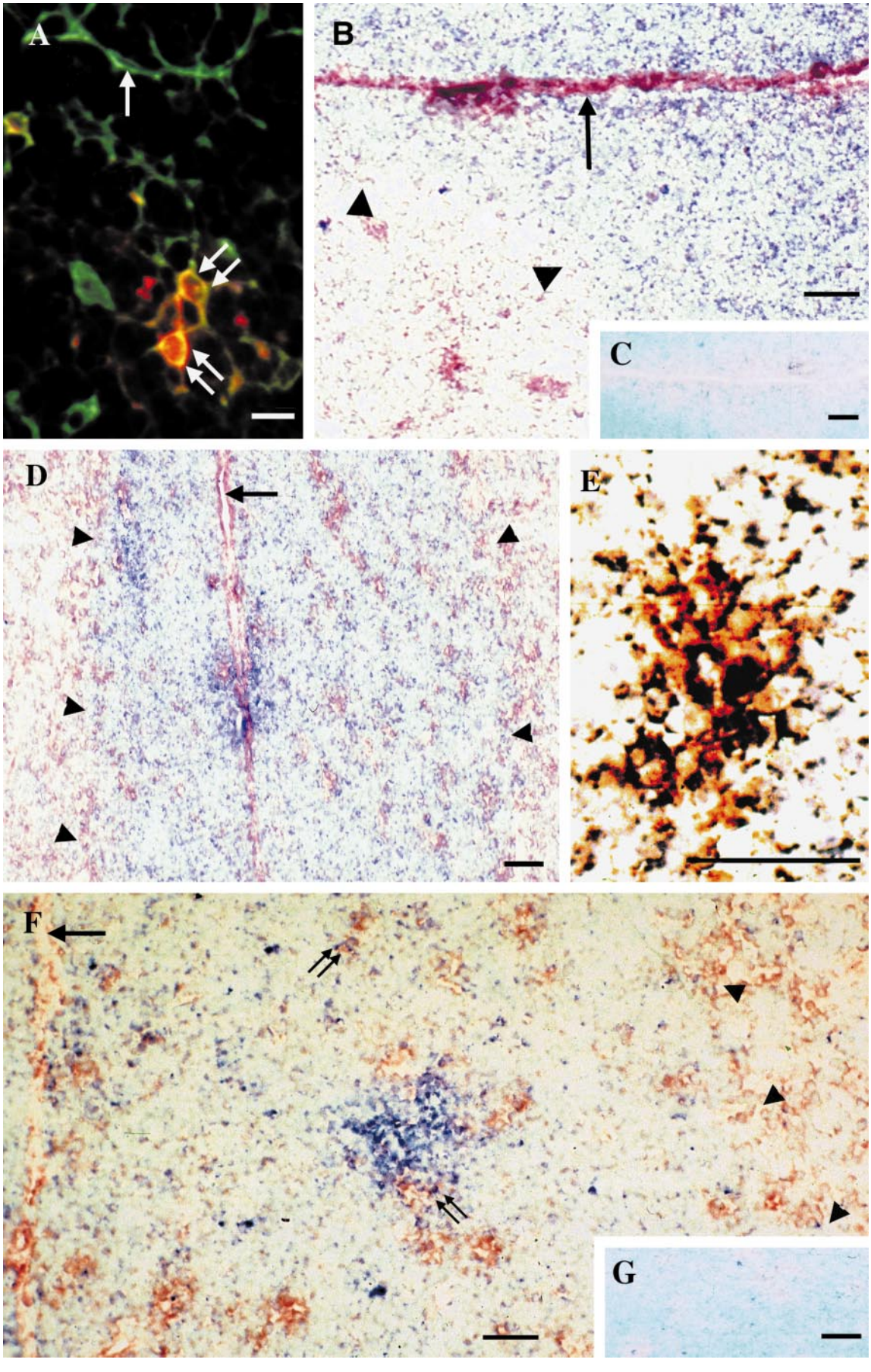
In situ hybridisation showed an almost uniform expression of $P2Y_2$ receptor mRNA in thymocytes of the cortex, but not in the medulla or other cells of the thymus (Fig. 5B,D). $P2Y_2$ receptor mRNA expression was often colocalised with protein expression of $P2X_1$ receptors (Fig. 5E) and $P2X_4$ receptors. No $P2Y_4$ mRNA expression was detected. $P2X_4$ receptor mRNA expression was seen in scattered cells throughout the cortex and in cell clusters. Colocalisation experiments for $P2X_4$ receptor mRNA and $P2X_4$ receptor immunoreactivity revealed that the $P2X_4$ receptor protein and mRNA were not always detectable in the same cell (Fig. 5F) and may indicate that the half-life of $P2X_4$ mRNA is quite short. $P2X_4$-receptor-immunoreactive thymocytes were also seen in cortex and medulla, whereas the expression of $P2X_4$ receptor mRNA was restricted to the cortex. Negative controls of the in situ hybridisation experiments revealed the specificity of the probes. After competition of DIG-labelled probe with a 75-fold excess of unlabelled probe, staining was greatly reduced and after omitting the labelled probe staining was completely abolished (Fig. 5C,G).

◀ **Fig. 4A–J** Immunoreactivities for P2X receptors on epithelial cells in the rat thymus. Immunohistology was carried out with the nickel-intensified DAB/peroxidase method, except in **C** where tyramide amplification was used. **A** Immunoreactivities for $P2X_2$ receptors in medullary epithelial cells (*m* medulla, *c* cortex). **B** $P2X_2$-positive medullary epithelial cells showing cell-cell contacts (*arrow*). **C** $P2X_3$-positive medullary epithelial cells (*arrowheads*) surrounded by thymocytes (*arrow*), shown with a counterstain for nuclei. **D** $P2X_2$-positive septal epithelial cells. **E** Preabsorption of $P2X_2$ antibody in a corresponding area in **D**. **F** Septal epithelial cells immunoreactive for $P2X_6$ receptors (*arrowheads* indicate the septum). **G** Epithelial cells in the outermost layer of Hassall's corpuscles, the centres indicated by *asterisks*, are $P2X_6$-positive. **H** Preabsorption of $P2X_6$ antibody in a corresponding area of **G**. **I** $P2X_7$-positive septal epithelial cells (position of the septum indicated by *arrows*). **J** Preabsorption of $P2X_7$ antibody in a corresponding area of **I**. *Scale bars* 60 µm **(A,D–J)**, 30 µm **(B,C)**

Discussion

Our Western blot analysis revealed specific detection of P2X receptor subunit proteins in crude thymic extracts. The detection of 70-kDa and 140-kDa P2X proteins is consistent with reports in the literature that describe the detection of P2X proteins of similar size. Sun et al. (1998) report a single band of 70 kDa; Scase et al. (1998) two bands of 60 kDa and 45 kDa. Berry et al. (1998) detected three bands of 160 kDa, 70 kDa and 50 kDa for $P2X_1$ in human heart. In HEK293 cells transfected with rat P2X receptors molecular weights between 57 kDa and 64 kDa have been found for $P2X_{2,3 \text{ and } 4}$ (Vulchanova et al. 1997; Lê et al. 1998). The bigger $P2X_7$ receptor was described to have a molecular weight of 70 kDa in transfected HEK293, CHO and NtW8 cells (Collo et al. 1997). With our antibodies for $P2X_{5 \text{ and } 7}$ a single band of 70 kDa was observed in Western blots of rat stratified squamous epithelia (Gröschel-Stewart et al. 1999). The same antibodies were also tested in Western blots of transfected cells (Oglesby et al. 1999). In this study a 58-kDa band for $P2X_1$ receptors was obtained, $P2X_2$ and $P2X_3$ receptors were recognised as doublets of approximately 60 and 64 kDa, $P2X_4$ receptors were detected at 60 and 64 kDa and with a band at approximately 120 kDa, $P2X_5$ receptors were detected with a single band of 64 kDa and with antibodies for $P2X_6$ and $P2X_7$ receptors bands of 50 and 95 kDa were obtained. All immunoreactivities could be preabsorbed after incubation of the antibody with its cognate peptide. We did not detect any P2X proteins smaller than 70 kDa in size in our experiments.

The molecular weights of 70 kDa and 140 kDa for each receptor subunit correspond with data from the literature, describing P2X receptors as highly glycosylated proteins that can form dimers (Vulchanova et al. 1997). These receptor proteins also undergo alternative splicing (Lê et al. 1997; Townsend-Nicholson et al. 1999) and the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presence of alternative spliced P2X receptor isoforms together with post-translational modifications such as glycosylation (Newbolt et al. 1998) gives rise to the potential for great variation in the size of the detected proteins.

P2X and P2Y receptors on thymocytes

Immunoreactivity for $P2X_1$ and $P2X_4$ receptors and expression of $P2Y_2$ receptor mRNA has been shown in thymocytes in this study.

Two lines of evidence have been brought forward, describing functional involvement of thymocyte P2X and P2Y receptors in mitogenic stimulation and apoptosis (Chow et al. 1997).

One line comes from the observation that extracellular ATP can cause cell death, probably through the opening of the membrane channel/pore $P2X_7$ (Di Virgilio et al. 1989). The other line is seen, however, in the participation of the $P2X_1$ receptor in mediating programmed cell death, since Valera et al. (1994) described extensive sequence homology between $P2X_1$ receptors and RP-2, a gene expressed during thymocyte apoptosis. Some authors have denied a role for P2X receptors in thymocyte activation (Jiang et al. 1996; Koshiba et al. 1997), while Ross et al. (1997) rejected involvement of G-protein-coupled purinoceptors in thymocyte apoptosis. However, Koshiba and co-workers (1997) found $P2Y_2$ mRNA upregulation after steroid hormone treatment and T-cell receptor cross-linking of mouse thymocytes. This situation became even more complicated after species differences between mouse and rat in $P2X_1$ mRNA upregulation after glucocorticoid administration were revealed (Koshiba et al. 1997). The in vitro stimulation of thymocytes with cortisol used in many of these studies gives no general indication for the onset of apoptosis, as pointed out by Nakamura et al. (1997). Cortisol-treated cells in vivo die by neglect and become pyknotic, apoptosis being a secondary event. The vast majority of apoptotic lymphocytes become phagocytosed directly by macrophages, so that there are no free necrotic lymphocytes.

The existence of $P2X_4$ mRNA in the thymus has been described by Bo et al. (1995). Our findings of $P2X_1$ in cortical and a minor portion in medullary thymocytes are consistent with the immunohistochemical data from Chvatchko et al. (1996), who also detected the inhibition of dexamethasone-induced cell death after application of suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) in cultured thymocytes. These are antagonists of $P2X_1$ and $P2X_3$ but can potentiate agonist responses of $P2X_4$ (Bo et al. 1995; Townsend-Nicholson et al. 1999). Whether there is collaboration between these purinoceptors resulting in the induction of cell death remains to be shown. In this model $P2Y_2$ did not seem to be involved in the prevention of thymocyte apoptosis. The application of suramin would have antagonised the actions of $P2Y_2$, whereas PPADS would have had little effect (Harden et al. 1998). However, since no difference has been detected between these two P2 receptor antagonists in preventing cell death, $P2Y_2$ receptors do not appear to mediate apoptosis. The localisation of $P2X_1$ and $P2Y_2$ receptors mainly in the cortex and the finding of $P2X_4$ staining in cortex and medulla may reflect contrasting functions in the medulla and synergistic or reverse effects in the cortex. Some authors have found biphasic rises of intracellular calcium after administration of ATP to T cells (Zheng et al. 1991; Ross et al. 1997) and heterogeneous response to ATP in broader populations (Chused et al. 1996). This is consistent with the differential expression of purinoceptors on lymphocytes that we found using immunohistochemistry and in situ hybridisation.

Our colocalisation experiments showed coexpression of $P2X_4$ mRNA and $P2X_4$ protein in a number of thymocytes, but there were also cells where only one of the two signals can be detected. This is puzzling, but could be explained by a very short half-life of the $P2X_4$ mRNA and a long half-life of the $P2X_4$ protein. In some thymocytes described in our study we might have detected a stage where $P2X_4$ mRNA is expressed, but not yet translated into quantities of protein that could be detected by immunohistochemistry. In other thymocytes, where sufficient $P2X_4$ protein has accumulated to be detectable immunohistochemically, most of the $P2X_4$ mRNA might already have been degraded. The $P2X_4$ gene is alternatively spliced (Lê et al. 1997; Townsend-Nicholson et al. 1999) and it is possible that isoform mRNAs exist that have very short half-lives in some thymic cells. To our

◀ **Fig. 5A–G** Colocalisation studies of P2X and P2Y receptor subtypes and a marker for epithelial cells. Studies were carried out using immunofluorescence **(A)** or histochemical methods with mRNA of P2Y or P2X receptors being visualised using alkaline phosphatase and BCIP/NBT as a stain (giving a *blue/black* precipitate) **(B–G)**. Immunoreactivities for P2X receptors were visualised using tyramide amplification and a DAB/peroxidase stain (giving a *brown* precipitate). Counterstains with Contrast green have been conveyed through, showing cortical areas in *blue/green*, whereas the medullae are clear. **A** $P2X_2$-positive medullary reticular-shaped cells (stained *red* with Texas red) are identical with epithelial cells (*double arrows*) stained with mouse monoclonal antibody against cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19 and FITC (*green*), but not all medullary epithelial cells (*single arrow*) express $P2X_2$. **B** Expression of $P2Y_2$ mRNA in cortical thymocytes (*blue*) on a slide showing immunoreactivity for $P2X_2$ receptors (*brown*) in the septum (*arrow*) and some medullary epithelial cells (corticomedullary junction indicated by *arrowheads*). **C** An area corresponding to the septum in **B** was processed for in situ hybridisation, but without labelled probe. No nonspecific staining was detectable. **D** $P2Y_2$ receptor mRNA expression in cortical thymocytes (*blue*) is sometimes colocalised with immunoreactivities for $P2X_4$ receptors (*brown*). $P2X_4$-positive thymocytes are also detectable in the medulla (*arrowheads* indicate the corticomedullary junction). The septal area (*arrow*) is stained brown by $P2X_4$-positive vascular smooth muscle. **E** Cortical thymocytes expressing $P2Y_2$ receptor mRNA (*black*) show colocalisation with $P2X_1$ receptor immunoreactivity (*brown*). **F** Cortical thymocytes expressing $P2X_4$ receptor mRNA (*blue*) show occasional colocalisation (*double arrows*) with thymocytes immunoreactive for $P2X_4$ receptors (*brown*). Expression of $P2X_4$ mRNA is only detectable in the cortex, whereas $P2X_4$-immunoreactive thymocytes are visible in cortex and medulla (*single arrow* thymic septum, *arrowheads* corticomedullary junction). *Scale bars* 40 μm **(A)**, 30 μm **(B–G)**

knowledge, colocalisation of $P2X_4$ mRNA and protein has not been carried out previously at the level of individual cells, so comparison of our results with work of other investigators is not yet possible.

We failed to detect the presence of $P2X_7$ receptors on T cells although this has been demonstrated pharmacologically (Chused et al. 1996). Buell et al. (1998) clearly demonstrated immunoreactivities for $P2X_7$ receptors on macrophages and dendritic cells in human tonsils, but we could not observe any staining for $P2X_7$ receptors on these cells in the thymus. However, this is consistent with the study by Collo et al. (1997) showing, by Northern blotting, that $P2X_7$ receptors are absent from the thymus of adult rat. This technique could probably not detect the very low $P2X_7$ expression of some epithelial cells shown by us in this study. Different expression in vivo and in cell culture, or alternative gene splicing, could also be a cause for discrepancies with other studies. Our P2X antibody is directed against a 15 amino acid portion at the C-terminus of this purinoceptor, which may not be present in some alternatively spliced $P2X_7$ isoforms.

P2X receptors in the vasculature

In vascular smooth muscle P2X receptors have been shown to mediate vasoconstriction. The receptors in the vasculature (α-adrenergic and P2X) can be excited by sympathetic innervation with noradrenaline and ATP as cotransmitters, leading to constriction of the vessels (Burnstock 1990).

The vessels of murine thymus are richly innervated by sympathetic, parasympathetic and sensory nerves (Williams et al. 1980; Weihe et al. 1991). Peptidergic immunomarkers show tyrosine hydroxylase and neuropeptide Y coexistence in sympathetic nerves, vasoactive intestinal polypeptide and peptide histidine isoleucine in parasympathetic neurons and calcitonin gene-related peptide and tachykinins (substance P and neurokinin A) in sensory nerves (Weihe et al. 1991). The $P2X_2$ receptor was mainly expressed in vascular smooth muscle of the larger septal vessels, the $P2X_1$ receptor was seen in small and big blood vessels of the septa and in the corticomedullary junction, whereas the $P2X_4$ receptor occurred predominantly on vascular smooth muscle of small vessels in cortical and medullary areas and in some septa. The vasoconstrictor tone of these vessels may be controlled by the sympathetic innervation that, in other sites, has been shown to contain both noradrenaline and ATP as well as neuropeptide Y (Burnstock 1990). $P2X_1$, $P2X_2$ and $P2X_4$ receptors differ in their pharmacology to extracellular ATP (Humphrey et al. 1998). The differential expression of P2X receptor subtypes in vascular smooth muscle of big or small vessels, or in the septa, cortical, or medullary areas may reflect physiological needs to differentially control the vascular tone at these sites. Although it was first thought that only $P2X_1$ receptors were prominent in smooth muscle (Collo et al. 1996), in a more recent study $P2X_{1,2\ and\ 4}$ receptors have been found in different regions of the vasculature (see Nori et al. 1998).

In the present study, $P2X_5$ receptors were found on vascular smooth muscle or fibroblasts and it was suggested that $P2X_3$ receptors occur on endothelial cells. So far, $P2X_5$ has been related to cell proliferation or differentiation (Gröschel-Stewart et al. 1999) and $P2X_3$ has been shown to be expressed largely in sensory neurons (Lewis et al. 1995).

P2X receptors on epithelial cells

Subcapsular and perivascular epithelial cells secrete chemoattractants recruiting lymphocyte precursors from the blood for intrathymic T-cell development (Kendall 1991). In our experiments we showed that such reticular-shaped cells express $P2X_2$ receptors. $P2X_2$-positive epithelial cells were present in cells along the septa. $P2X_6$ and $P2X_7$ receptors were also found on septal epithelial cells but were seen less frequently than $P2X_2$-positive cells.

The majority of nerves (sympathetic, parasympathetic and sensory) invade the thymus through the septa travelling along with the vessels (Kendall 1991). Noradrenergic fibres course into the thymus at these sites, branching from the septa into the cortex (Felten and Felten 1989). Nerve fibres can make contacts with reticular-shaped cells (like epithelial cells) as demonstrated at the light-microscopic and ultrastructural levels (Novotny et al. 1990), offering the possibility of a noradrenergic and purinergic stimulation of subcapsular, perivascular and septal epithelial cells.

Another area of extensive noradrenergic innervation and with broad vascular branching exists at the corticomedullary junction (Felten and Felten 1989). Tyrosine-hydroxylase-positive and noradrenergic fibres have been demonstrated to make close contacts to murine macrophages and mast cells (Williams et al. 1980; Weihe et al. 1991), whereas medullary epithelial cells seem to be innervated mainly by sensory nerves (Novotny et al. 1990). However, in cell culture studies β-adrenoceptors could be detected on perivascular, septal and medullary epithelial cells, indicating a role for sympathetic innervation of medullary epithelial cells (Kurz et al. 1997). We have shown the presence of $P2X_{2,3\ and\ 6}$ receptors on medullary epithelial cells. These cells could be stimulated via sympathetic nerves costoring noradrenaline and ATP or by other cells of the thymic microenvironment, which have yet to be shown to release ATP.

Physiologically medullary epithelial cells are important for the synthesis of neurophysin, oxytocin, arginine vasopressin (AVP), somatostatin, interleukin-1 and -4, colony-stimulating factor for macrophages and thymic hormones (Kendall 1991). AVP, somatostatin, cytokines and thymic hormones have been shown to effect thymocyte proliferation in several ways, and AVP and oxytocin potently act on blood vessels.

The $P2X_3$ receptors on medullary epithelial cells may form dimers with $P2X_2$ receptors. Dimerisation of $P2X_2$

and $P2X_3$ receptors has been claimed previously in sensory neurons (Lewis et al. 1995). No clear evidence could be found for $P2X_3$ receptors on nerve fibres.

The function of Hassall's corpuscles, which are formed from specialised epithelial cells in the medulla, where we detected $P2X_6$ staining, is not elucidated yet. However, secretion of thymic hormones and opioids has been associated with them (Ritter and Crispe 1992).

In conclusion we found immunoreactivities for P2X receptors at three sites that play key roles in the system of lymphocyte travel, maturation and sorting. Our results suggest that the preponderance of P2 receptors in thymic structures may reflect a significant role for ATP as an extracellular messenger in the thymus. Further research in the field will help to elucidate the molecular mechanisms underlying the role of these receptors in the thymus.

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