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Ultrastructural Identification of $P2Y_2$ Receptor mRNA in the Rat Thymus

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Key Words
$P2Y_2$ receptor · Thymus · Hybridisation, in situ · Rat (Sprague-Dawley)

Abstract
In situ hybridisation to identify $P2Y_2$ receptor mRNA was performed for the first time at the ultrastructural level on the thymus of adult male rats. These studies revealed transcripts for $P2Y_2$ receptors in cortical T cells and endothelial cells of thymic blood vessels. These transcripts are likely to be linked with the production of functional $P2Y_2$ receptors in these cells. In the T cells, transcripts for the $P2Y_2$ receptor were localised in the cytoplasm as well as on the smooth endoplasmic reticulum and cell membrane. Dividing T cells also expressed $P2Y_2$ receptor mRNA, mostly in the cytoplasm around chromosomal material. Endothelial cells displaying labels for $P2Y_2$ receptor transcripts were of cortical arteries/arterioles and capillaries and of postcapillary venules in the corticomedullary junction. $P2Y_2$ mRNA transcripts were localised in the cytoplasm of endothelial cells, although they did not appear to be specifically associated with subcellular organelles or structures. In postcapillary venules, T cells displaying labelling for the $P2Y_2$ receptor were seen migrating across the $P2Y_2$ receptor mRNA-positive endothelium. Our findings are discussed in terms of the relationship between thymic immune cells and the endothelium. This includes the issue of immune cell trafficking into the circulation, and the ATP-related regulatory role and involvement of $P2Y_2$ receptors in the rat thymus.

Abbreviations used in this paper

DIG	digoxigenin
PBS	phosphate-buffered saline
SSC	saline sodium citrate buffer

Introduction

The roles of extracellular ATP in various cell systems including vascular endothelial cells, epithelial secretory/endocrine cells in various organs and cells of the immune system have been established over the last few decades [Burnstock, 1972, 1995; Fredholm et al., 1997; North and Barnard, 1997; Abbracchio and Burnstock, 1998]. Seven subtypes of P2X receptors, a family of ligand-gated cation

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channels, and six subtypes of P2Y receptors, a family of G protein-coupled P2Y receptors, are now recognised in mammals [Abbracchio and Burnstock, 1994; Burnstock and King, 1996; Ralevic and Burnstock, 1998].

The presence of both P2X and P2Y receptors has been reported in the thymus, on immune cells as well as blood-related cells [Di Virgilio et al., 1989; Valera et al., 1994; Collo et al., 1997; Koshiba et al., 1997; Glass et al., 2000]. P2X receptors have been found to participate in controlling mitogenic stimulation of thymocytes, cytokine release from macrophages, formation of macrophage polykarions and cytotoxicity [Collo et al., 1997] and induction of apoptosis in thymocytes [Valera et al., 1994]. Mitogenesis and apoptosis also involves P2Y receptors [Valera et al., 1994; Chow et al., 1997]. Importantly, the $P2Y_2$ receptor in T cells is expressed as an immediate early gene after T cell receptor triggering [Koshiba et al., 1997]. Therefore, it has been concluded that the expression of $P2Y_2$ receptors is tightly regulated during the process of T cell sorting, i.e. in induction of clonal expansion or apoptosis. More recently, $P2Y_2$ receptors have been demonstrated in thymic epithelial cells in vitro [Bisaggio et al., 2001]; however, another report has highlighted the importance of using intact thymus when studying function [Kendall, 1991].

There is evidence for the presence of P2Y receptors on endothelial cells in thymic blood vessels [Glass et al., 2000]. P2Y receptors in the systemic blood vessels are known to control vasomotor functions via endothelial $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor subtypes [Ralevic and Burnstock, 1998]. The organisation of the vascular system of the thymus allows observation of T cell migration to and from the thymus. Small branches of the thoracic and thyroid arteries enter the thymus mostly via interlobular septa. In the corticomedullary junction these arteries give rise to small arterioles and capillary loops to supply cortex and medulla [see Weiss, 1983; Kierszenbaum, 2002]. The endothelium of cortical capillaries is usually 'non-penetrable' for T cells, whereas T cells can penetrate the post-capillary venules of the corticomedullary junction.

In the present study we investigated the expression of transcripts of $P2Y_2$ receptors at the ultrastructural level in the rat thymus, in situ, including resting and migrating T cells as well as vascular endothelial cells. Due to the tight regulation of $P2Y_2$ receptor mRNA in T cells [Koshiba et al., 1997], the results from the present study are likely to indicate the presence of functional $P2Y_2$ receptors. Results from this study give a first clue for participation of purinergic signalling in controlling T cell migration to and from the thymus.

Methods

Animals

Breeding, maintenance and killing of the rats used in this study followed principles of good laboratory animal care and experimentation in compliance with UK national laws and regulations. Tissues were taken from 3-month-old male Sprague-Dawley rats (n = 6).

Tissue Preparation

Rats were anaesthetised with sodium pentobarbitone (60 mg kg^{-1} i.p. Sagatal, RMB Animal Health, Harlow, UK) and perfused through the heart (left ventricle) with fixative containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 mol/l phosphate buffer at pH 7.4 for 15 min (at room temperature). The thymi were dissected out and placed in the same fixative for 5 h at 4°C, and then transferred to phosphate buffer and stored overnight at 4°C. The following day, cross sections of 50–60 μm through the median part of the thymus were cut on a Vibratome and collected in sterile 0.1 mol/l Tris-buffered saline. Sections were then processed for pre-embedding in situ hybridisation of mRNA $P2Y_2$ receptor for electron microscopy.

In situ Hybridisation

To improve the infiltration of reagents into thymus sections, these were exposed to 30% ethanol for 15 min at room temperature. Sections were then rinsed in phosphate-buffered saline (PBS) and incubated in prehybridisation buffer [consisting of 50% formamide, 2 × saline sodium citrate buffer (SSC), 1 × Denhardt's (BFP, Sigma), 1 mg/ml sheared and denatured salmon sperm DNA (Sigma) and 1 mg/ml tRNA type X from baker's yeast (Sigma)] for 1 h at 37°C in a humidified chamber as previously described [Glass et al., 2000]. This was followed by incubation in hybridisation buffer consisting of 1 ng digoxigenin (DIG)-labelled probe (see below) in 1 μl prehybridisation buffer at 37°C overnight. Washing of the unhybridised probe was performed 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 probe was detected by overnight incubation (initially at room temperature, then at 4°C) of sections with the sheep anti-DIG antibody conjugated to 1-nm gold particles (Boehringer Mannheim, Germany) diluted 1:30 in PBS containing 0.1% sodium azide. This was followed by several washes in PBS, fixation for 10 min with 1% glutaraldehyde (in 0.1 mol/l phosphate buffer, pH 7.4) and washing in phosphate buffer. Before application of the silver enhancement procedure, sections were washed 10 × 5 min in deionised distilled water (to remove all ions, especially chloride). The hybridisation reaction was then enhanced with silver (in a dark chamber for 10–16 min at room temperature) using a silver-enhancing kit (British Biocell Int., Cardiff, UK). After washing in deionised, distilled water and 0.1 mol/l cacodylate buffer (pH 7.4), sections were postfixed with 1% osmium tetroxide (in cacodylate buffer), dehydrated in a graded series of ethanol and flat-embedded in Araldite. The ultrathin sections were cut from the thymus stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope.

Probe

Rat $P2Y_2$ antisense oligonucleotide probe (of 45 nucleotides involved: 5′-GATGGCGTTGAGGGTGTGGCAACTGAGGTC-AAGTGSTCGGAAGGA-3′ [Glass et al., 2000]; for rat $P2Y_2$

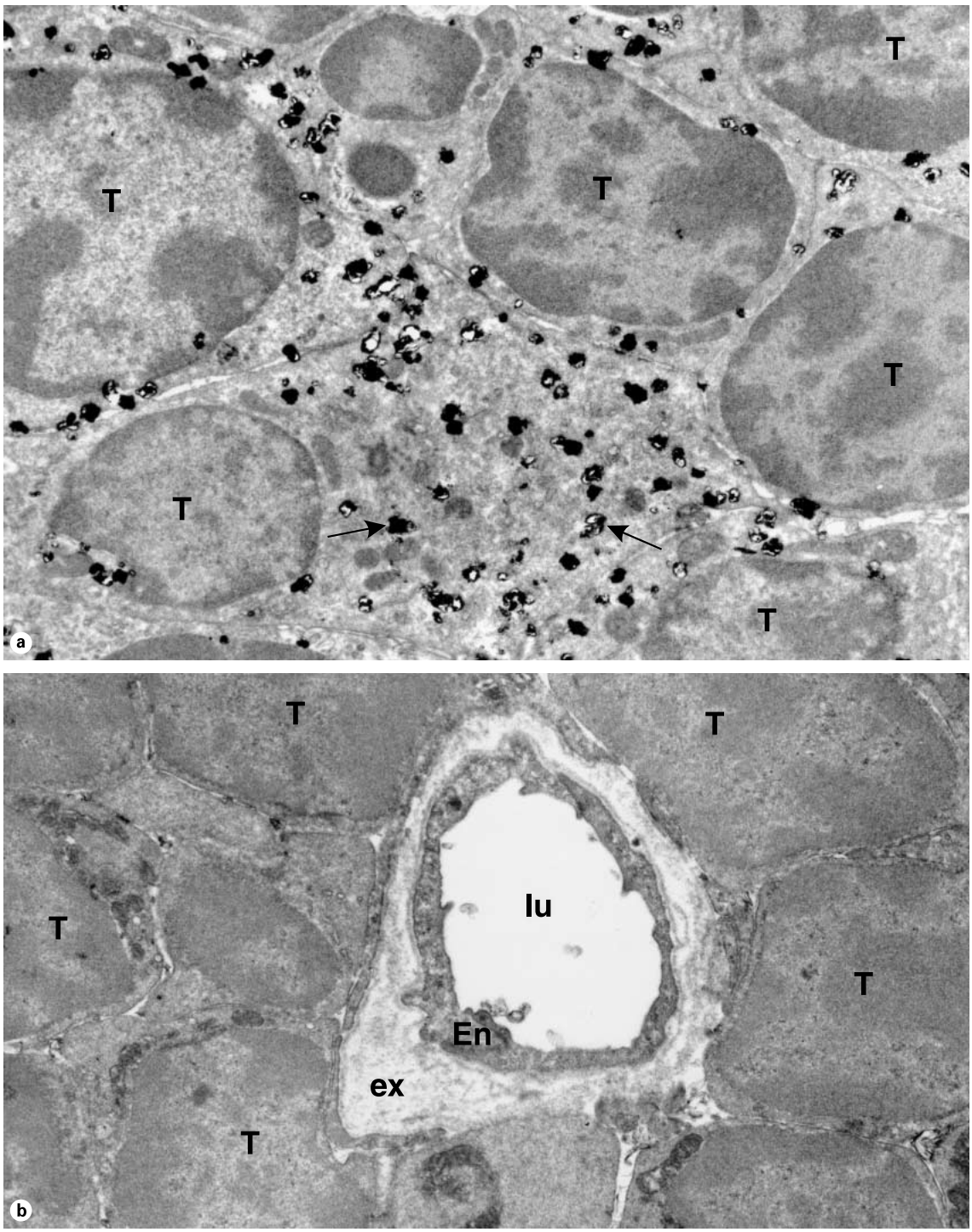


Fig. 1. Ultrastructural identification of $P2Y_2$ receptor mRNA in the cortex of rat thymus. a Note intense labelling (numerous 'black' gold-silver grains: arrows) localised in the cytoplasm of resting T cells (T) of the specimen that was hybridised to the DIG-labelled rat $P2Y_2$ receptor antisense oligonucleotide probe. b A control specimen, processed for in situ hybridisation with an excess of unlabelled probe added to the DIG-labelled probe (competition of sense and antisense probes), demonstrates lack of labelling for $P2Y_2$ receptor mRNA in the T cells as well as in vascular endothelium (En) of a cortical capillary. lu = Lumen; ex = extracellular matrix of perivascular space. a ×11,000. b ×11,000.

receptor was obtained from MWG Biotech (Germany). It was labelled at its 3′-end with the DIG oligonucleotide tailing kit (Roche Diagnostics) according to the manufacturer's instructions. The specificity of the probe was checked by comparison of the sequence with the SwissProt Database and was found to be highly specific for rat $P2Y_2$ receptors.

Controls for in situ Hybridisation

Negative controls included omitting the probe, use of labelled 'sense' probe and competing labelled 'antisense' probe with a 75-fold excess of unlabelled 'antisense' probe [Glass et al., 2000].

Results

Using a DIG-labelled rat $P2Y_2$ antisense oligonucleotide probe, $P2Y_2$ receptor mRNA was identified in the rat thymus by the pre-embedding in situ hybridisation method for electron microscopy (fig. 1a). In contrast, no $P2Y_2$ receptor mRNA was detected in control preparations when a sense probe was used or a step with labelled antisense probe was omitted. The signal was also greatly reduced after competing labelled probe with unlabelled probe on the tissue sections (fig. 1b). In in situ hybridised preparations, $P2Y_2$ receptor mRNA was identified in thymic immune cells, thymocytes (T cells) of the cortex (fig. 1a, 2), and in endothelial cells of thymic blood vessels (fig. 3, 4). In T cells, $P2Y_2$ receptor mRNA was primarily localised in the cytoplasm (fig. 1a, 2a, b). The cytoplasmic expression of transcripts also concerned T cells undergoing mitotic divisions (fig. 2a). However, in dividing cells, transcripts were prevalent in the peripheral cytoplasm. In addition to the cytoplasmic expression of $P2Y_2$ receptor mRNA, transcripts were also related to smooth endoplasmic reticulum and cell membrane. This was particularly apparent in T cells of a large size in specimens exposed to silver enhancement for a shorter time (fig. 2b).

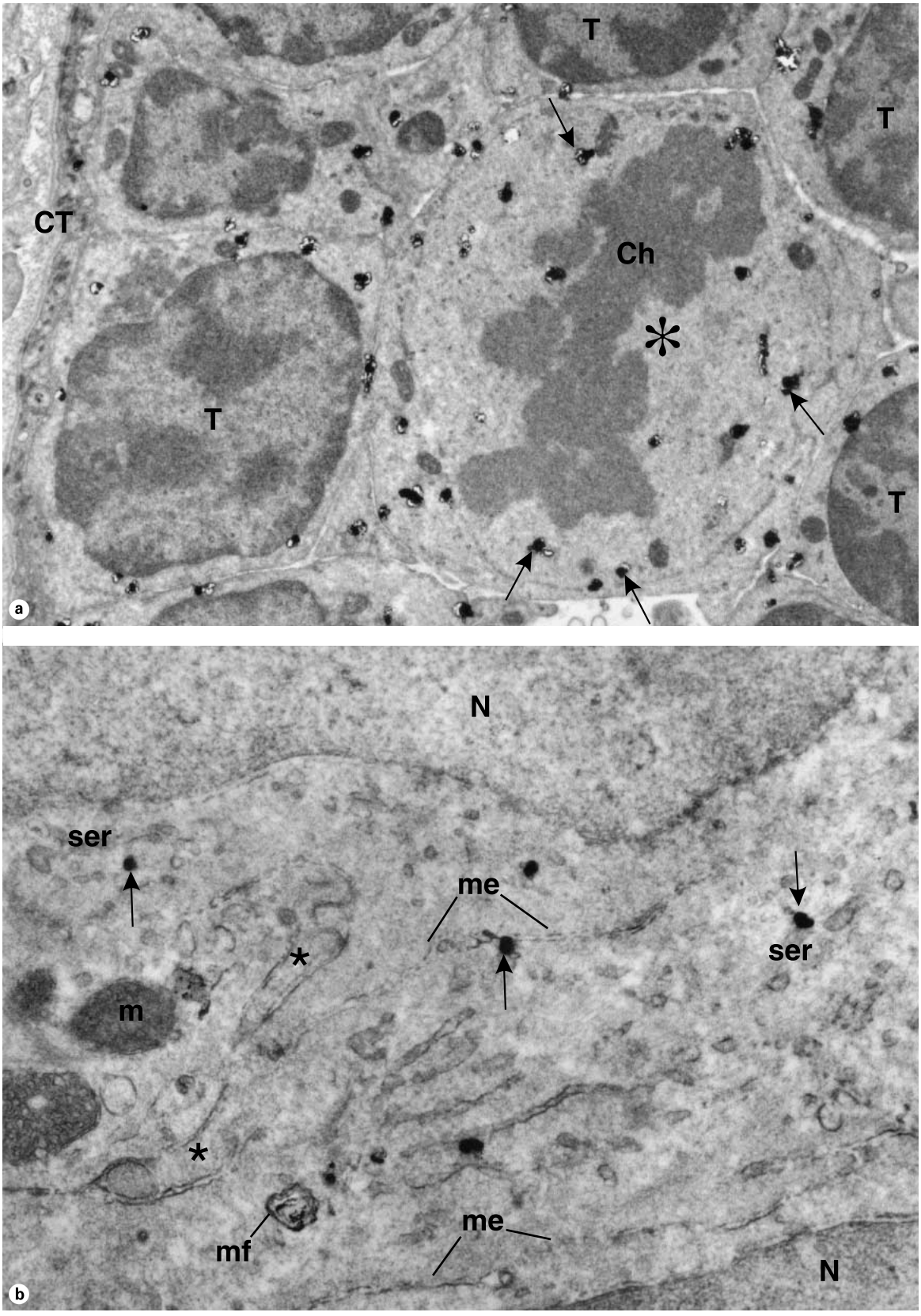
Labelling for $P2Y_2$ receptor transcripts in endothelial cells was observed in vessels of various diameters and locations throughout the thymus. In the cortex and corticomedullary junction, arteries, arterioles and capillaries were usually labelled (fig. 3a, b). Unlabelled endothelial cells were at times also observed neighbouring with $P2Y_2$-positive immune cells of the cortex and corticomedullary junction (fig. 3c). At the junction of cortex and medulla, the endothelium of postcapillary venules was usually richly labelled (fig. 3d). Mature T cells migrating across the $P2Y_2$ receptor mRNA-positive endothelium of postcapillary venules were observed (fig. 3e). Other thymic blood vessels similar to those spreading through the connective tissue of the septa also displayed endothelial cells positive for $P2Y_2$ receptor mRNA (data not shown). Within a single vessel, the intensity of the labelling for endothelial $P2Y_2$ receptor transcripts varied from one cell to another (fig. 3b). $P2Y_2$ receptor mRNA-positive endothelial cells of vessels of greater diameter, such as cortical arteries or arterioles, were at times pronounced and bulged into the vessel lumen (fig. 3a). In endothelial cells expressing $P2Y_2$ receptor mRNA, the labelling was detected in various regions of the cytoplasm but did not appear to be associated specifically with endoplasmic reticulum, Golgi apparatus or mitochondria. This is exemplified by the endothelium in figure 4 at high magnification.

Discussion

The present electron microscopy study demonstrates that $P2Y_2$ receptor mRNA is expressed in cortical T cells and vascular endothelial cells of the rat thymus. Transcripts may also be present in some epithelial cells. These results extend previous data from our laboratory regarding the presence, at the light microscope level, of $P2Y_2$ receptors in rat thymus [Glass et al., 2000] and provide new insights into the expression of purinergic receptor mRNA in this gland at the ultrastructural level. Our findings agree with the view that P2 receptors play an important role in the immune system [see Burnstock, 2001].

The pre-embedding in situ hybridisation procedures applied for the electron-microscopic purposes in this study allowed good morphological-ultrastructural preservation of the thymic immune tissue and blood vessels. Good tissue preservation was likely to be due to the effect of omission of proteolytic digestion of the Vibratome sections with proteinase K, which usually is used for the in situ hybridisation protocol at the light microscope level.

Fig. 2. Ultrastructural identification of $P2Y_2$ receptor mRNA in the superficial cortex (a) and corticomedullary region (b). a Among labelled T cell profiles (T) note a profile (large asterisk) undergoing cell division (anaphase); the labels expressing $P2Y_2$ receptor mRNA (arrows) are mostly localised in peripheral cytoplasm. Ch = Chromosomes; CT = interlobular connective tissue. b Magnified fragments of large T cells with visible nuclei (N); note localisation of $P2Y_2$ receptor mRNA labels (arrows) associated with the smooth endoplasmic reticulum (ser) and cell membrane (me). Processes from a thymic epithelial cell (small asterisks) stretch between the T cells; it is likely that some membrane-associated labelling is related to epitheliocytes. m = Mitochondria; mf = myelin figure. The specimen in b was enhanced with silver for 11 min, whilst that in a for 15 min. a ×11,000. b ×34,000.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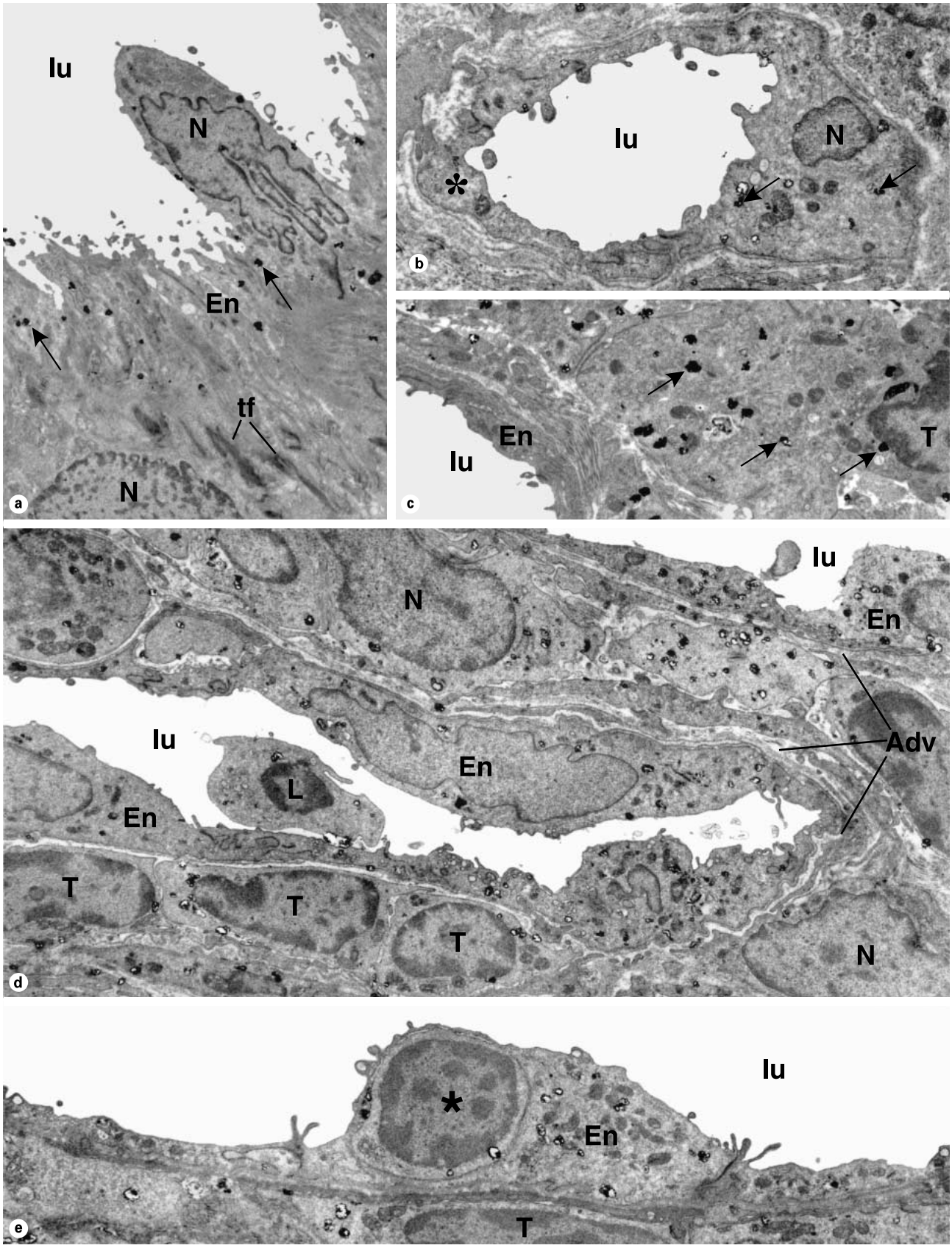
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According to Matsuno et al. [2000], the pre-embedding in situ hybridisation methodology appears to be better than the postembedding procedures with respect to preservation of mRNA. Ultrastructural in situ hybridisation studies of various tissues/organs demonstrate that mRNA can be localised primarily in the cytoplasm [Copray et al., 2000]. However, ultrastructural distribution of transcripts within the cell interior may strictly depend on cell type and mRNA examined, for example, localisation of Xcat2 mRNA in germinal granules during oogenesis [Kloc et al., 2000]. It is therefore considered that the combination of electron microscopy and in situ hybridisation is an important tool for clarifying the intracellular localisation of a specific mRNA and the site of synthesis and hence cell physiological state and function [Matsuno et al., 2000].

The present study demonstrates that the majority of immune cells examined displayed cytoplasmic localisation of the $P2Y_2$ receptor transcripts. This includes thymocytes undergoing the cell division or mature T cells migrating across venules. The transcripts in the T cells of larger size were additionally related to the endoplasmic reticulum and cell membrane. The cytoplasmic localisation of $P2Y_2$ receptor mRNA was also detected in thymic vascular endothelial cells. Cytoplasmic localisation of transcripts for $P2Y_2$ receptor mRNA in the T cells is not surprising since these cells are usually scarce in subcellular organelles and structures including endoplasmic reticulum. Our results therefore suggest that in the majority of T cells examined, $P2Y_2$ receptor proteins are synthesised mainly in the cytoplasm (possibly on the free ribosomes). A similar conclusion may be drawn with respect to the thymic endothelial cells, which in contrast contain intracellular organelles and structures such as endoplasmic reticulum or Golgi apparatus that could be implicated in receptor synthesis. The possible roles of P2 receptors, including $P2Y_2$ receptors in the physiology of thymic immune cells and vascular endothelium, are discussed below in more detail.

It is known that extracellular purines together with their receptors take part in immunoregulation [Sitkovsky, 1998]. Evidence suggests that during immune cell maturation or response to inflammatory mediators, the expression of P2 receptors is altered in these cells (upregulation and downregulation of the receptor) [Dubyak et al., 1996; see Burnstock, 2001]. During monocyte to macrophage maturation, for example, a decrease in $P2Y_2$ receptor mRNA has been observed simultaneous to increased expression of $P2X_7$ receptor mRNA [Dubyak et al., 1996]. Coupling of the P2Y receptor to a signal transduction mechanism may be crucial for cellular proliferation and differentiation [Neary, 2000]. A role for the $P2Y_2$ receptor in mitogenesis and mediation of proliferation has been suggested [Tu et al., 2000]. Our results imply that $P2Y_2$ receptors are produced by dividing T cells, maturing T cells and T cells entering the circulation. It seems therefore that the role for $P2Y_2$ receptors on immune cells is diverse and perhaps crucial to the physiological function and fate of T cells in the thymus.

It is clear in the present study that $P2Y_2$ receptor mRNA transcripts are present throughout the thymic vascular system, but the intensity of expression is variable. The role of P2Y receptors present on endothelial cells is well established. These receptors control local blood flow by mediating vasodilatation [Ralevic and Burnstock, 1991; Purkiss et al., 1994; Bowden et al., 1995; Henderson et al., 1995; Burnstock, 1996; Gödecke et al., 1996; Patel et al., 1996]. P2Y receptors mediate vasodilatation by Ca^{2+}-dependent activation of nitric oxide synthase and production of nitric oxide and an endothelium-derived hyperpolarising factor [Burnstock and Ralevic, 1994; Vanhoutte, 2000]. The presence and pharmacological action of $P2Y_2$ receptors on endothelial cells have been identified in rabbit aorta [Chinellato et al., 1994], rat coronary microvessels [Gödecke et al., 1996] and rat mesenteric arteries [Ralevic and Burnstock, 1996; Ziyal, 2002].

The demonstration that endothelial P2Y receptors (at least $P2Y_1$-like receptors on pulmonary artery endothelium) stimulate adherence of leukocytes in cell culture

Fig. 3. Ultrastructural identification of $P2Y_2$ receptor mRNA in the thymic blood vessels. a An arteriole at the corticomedullary junction displays endothelial (En) localisation of labelling for $P2Y_2$ receptor mRNA (arrows). No labelling in underlying cells is seen. N = Nucleus; tf = tonofilaments; lu = vessel lumen. b A capillary in the cortex (the blood-thymus barrier) shows labels for $P2Y_2$ receptor transcripts located in the endothelial cell(s); also note neighbouring endothelial cell profile that is free of labeling (asterisk). c A fragment of a corticomedullary capillary is not labelled for $P2Y_2$ receptor mRNA in the endothelium, although the perivascular T cells (T) are labelled (arrows). d A postcapillary venule in the corticomedullary junction (no blood-thymus barrier) displays an abundance of labelling for $P2Y_2$ receptor mRNA, both in the endothelial cells lining the venule and in the numerous mature T cells (of various shape) occupying perivascular space/adventitia (Adv), where T cells are probably moving toward the lumen. Labelled mature T cell (L = lymphocyte) in the lumen of the venule is also seen. e Note a mature T cell (asterisk) migrating across the endothelium into the lumen of a postcapillary venule; note that both endothelium and the migrating T cell are positive for $P2Y_2$ receptor mRNA. a ×6,000. b ×10,000. c ×8,500. d ×7,000. e ×10,000.

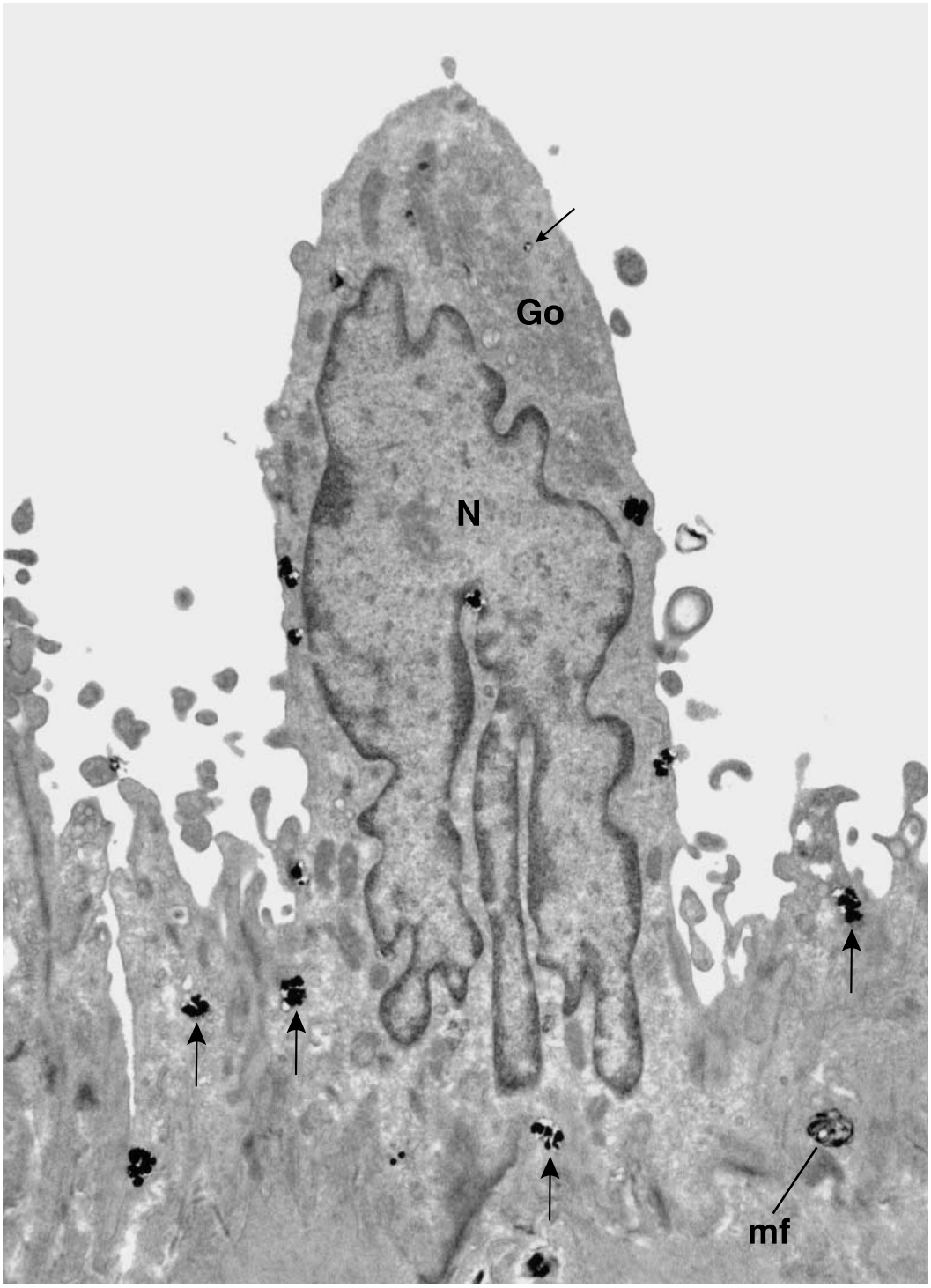

Fig. 4. A magnified fragment of labelled endothelium shown in figure 3a displaying cytoplasmic localisation of $P2Y_2$ receptor mRNA. Note that labelling is localised in the cytoplasm at the base of an endothelial cell (large arrows) as well as around the centrally located cell nucleus (N); some minor labelling is also seen in the vicinity of the Golgi apparatus (Go; small arrow). mf = Myelin figure. ×15,500.

experiments [Dawicki et al., 1995] also suggests other roles for these receptors on vascular endothelium, e.g. the involvement in immune cell trafficking via the vascular wall. The present results clearly demonstrate that endothelial cells of thymic blood vessels express $P2Y_2$ receptor mRNA. T cells crossing postcapillary venules at sites where no blood-thymus barrier exists were also $P2Y_2$ receptor mRNA-positive. It is well established that the majority of maturing T cells enter the circulation in the medulla and corticomedullary junction via postcapillary venules. The endothelium of the corticomedullary postcapillary venules was more intensely labelled for $P2Y_2$ receptor mRNA than the endothelium of other thymic vessels, e.g. of cortical arterioles or capillaries. This suggests an increased presence of $P2Y_2$ receptors (or demands for the receptors) on endothelial cells of postcapillary venules as compared to other thymic vessels. Thus, it is likely that endothelial $P2Y_2$ receptors on postcapillary venules fulfil a role during T cell trafficking into the circulation, in addition to the other role(s) these receptors play.

In conclusion, it has been shown at the ultrastructural level that transcripts for $P2Y_2$ receptors are expressed in the cytoplasm of dividing, maturing and migrating T cells as well as in vascular endothelial cells. These findings suggest a sustained synthesis of $P2Y_2$ receptor proteins in these cells, and a sustained demand for the receptor, e.g. during T cell division in the cortex or passage of mature T cells through the endothelium into the circulation in postcapillary venules. The introduction of ultrastructural examination may be utilised in other situations where the precise cellular identification of mRNA is desirable, and in particular to study the changes that occur in pathological situations.

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