

Presence of the P2X₇ purinergic receptor on immune cells that invade the rat endometrium during oestrus

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Abstract

Eosinophils, macrophages and other leucocytes invade the uterine endometrium during oestrus and play a role in the tissue remodeling and immune responses that occur prior to implantation of the fertilized ovum. Adenosine 5'-triphosphate (ATP) and its metabolites influence uterine function via ATP receptors. In this study, we investigated the presence and localisation of the P2X₇ nucleotide receptor in the cells that infiltrate the uterine endometrium of adult female rats during oestrus at the electron microscope level, using gold–silver pre-embedding immunocytochemical techniques. P2X₇ receptor expression was found in the cytoplasm and the cell membrane of eosinophils, macrophages and fibroblasts in the endometrium during oestrus. These results suggest that ATP-mediated responses may be important in uterine preparation and remodeling before implantation and that this may involve several types of cells. In particular, the presence of P2X₇ receptors on endometrial stromal cells may indicate their involvement in apoptosis and immune and inflammatory responses.

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1. Introduction

Implantation of the fertilized ovum within the uterine wall in mammals is ensured by the close integration of fertilisation with events that prepare the uterus to receive the embryo. Under the influence of pituitary and ovarian hormones, cyclic changes occur in the uterus of sexually mature females (Crowley, 1974). In rats, this oestrus cycle occurs every 4–5 days and is divided into prooestrous, oestrus, metoestrus and dioestrus. Oestrus is the time of ovulation and lasts for approximately 24 h. During this time, the endometrium thickens and the uterus is prepared for implantation of the fertilised ovum (Zarrow et al., 1964). At the end of oestrus, if pregnancy has not occurred, a large amount of uterine tissue is shed to prepare for the next cycle. Study of mechanisms controlling endometrial growth, remodeling and regression has generated much interest and is relevant to understanding of uterine function.

A prominent event occurring during oestrus is the accumulation of a large number of blood cells (eosinophils, neutrophils, macrophages, lymphocytes and plasma cells) in the endometrium of rats (Lee, 1982; Tassell et al., 2000) and other mammals (Padykula, 1976; McMaster et al., 1992; Wang et al., 2000). These cells release a number of potent regulators and play an important role in uterine growth, remodeling (Luque et al., 1997) and immunity (Wang et al., 2000).

Adenosine 5'-triphosphate (ATP) and its metabolites are extracellular signalling molecules released from several cell types under physiologic and pathophysiologic conditions (Gordon, 1986; Burnstock, 2001) and there is evidence to show that ATP influences uterine functions (Gorodeski and Goldfarb, 1997). The effects of ATP are mediated by interactions of the nucleotide with effector cells through cell surface receptors called P2 purinergic receptors linked to specific signal transduction pathways (Abbracchio and Burnstock, 1998; Ralevic and Burnstock, 1998). These receptors, when activated by purines, perform several important cellular functions. We are particularly interested in the P2X₇ ATP receptor that plays a role in apoptosis, development of immunity and control of microbial infection (Di Virgilio, 1995; Coutinho-Silva et al., 2001; Di Virgilio et al., 2001). The role of P2X₇ receptors on cells of the uterus during oestrus is discussed in relation to the role of the endometrium during embryo implantation. Here, immunofluorescence and electron microscopic immunocytochemical techniques have been applied to examine the presence of P2X₇ receptor protein in the endometrium during rat oestrus.

2. Materials and methods

2.1. Animals

Principles of good laboratory animal care were followed, and animal experimentation was in compliance with specific national laws and regulations of the Home Office (UK) covering Schedule One procedures in accordance with the Animals (Scientific Procedures) Act, 1986. A total of 25 female Sprague-Dawley rats (approximately 250 g) were used in the study. Stages of the oestrus cycle were determined by vaginal smear; cycles were established for a minimum of 2 weeks before the animals were used. The rats were killed by

asphyxiation with CO₂ and cervical dislocation and the two uterine horns were removed. Uterine tissue was taken from the uterine horns and processed for immunofluorescence, electron-immunocytochemistry and standard electron microscopy.

2.2. Electron microscopy

For standard electron microscopy, uterine tissues were immersion-fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for 2 h, washed in stock buffer, post-fixed in 1% osmium tetroxide (in stock buffer) for 1 h at 4 °C, washed in 0.1 M sodium acetate buffer (pH 7.4), block stained with 2% uranyl acetate (in sodium acetate buffer) for 30 min at 4 °C, dehydrated in graded concentrations of ethanol and embedded in Araldite. Ultrathin sections were cut at 80 nm thickness, stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope.

2.3. Immunofluorescence-confocal microscopy

Fresh uterine tissues from six rats (three non-oestrus controls and three in oestrus) were imbedded in O.C.T. compound (BDH/Merk, Leicester, UK) and fast frozen in precooled (by liquid nitrogen) isopentane. Using a Reichert-Jung CM1800 cryostat, uterine tissues were cross-sectioned (at least five sections per animal) at 12 µm and collected on gelatin-coated slides and then fixed for 10 min in acetone. Sections were then (i) washed in phosphate-buffered saline (PBS), placed for 1 h in 5% non-immune normal goat serum (NGS) (Nordic Immunology, Tilburg, The Netherlands), (ii) incubated overnight with a polyclonal antibody to P2X₇ receptors (Roche Palo Alto, CA, USA) at a dilution 3 µg/ml of PBS containing 1% NGS and 0.1% sodium azide, (iii) washed in PBS, (iv) incubated for 90 min with Aleza Fluor 568 goat anti-rabbit IgG (H and L) (Leiden, Netherlands) diluted 1:400 in PBS, (v) washed in PBS, and (vi) mounted in Citifluor for examination with a fluorescence confocal microscope (BioRad 2001, Radiance system). Fluorescence filters at 568 nm excitation line, and objective lenses with 10× and 20× or 40× (oil immersion), were used for examination of the specimens. Consecutive individual images were collected at 2 µm intervals and stored digitally. Sections were also examined using a Zeiss Axioplan microscope (Zeiss, Germany) with a Leica DC200 digital camera.

2.4. Electron-immunocytochemistry

Uterine tissues from six rats (three controls and three in oestrus) were immersion-fixed at 4 °C for 2 h in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), washed in 0.05 M Tris-buffered saline (TBS) at pH 7.6, embedded in albumin and sectioned at 50–70 µm thickness on a vibratome. To detect P2X₇ receptor protein, sections were processed for the pre-embedding immunogold–silver labeling technique (see Loesch and Burnstock, 2001) using a polyclonal anti P2X₇ receptor antibody (Roche Palo Alto). After blocking non-specific protein binding sites with 10% non-immune NGS, the sections were incubated for 24–30 h at room temperature with the P2X₇ antibody (at a dilution 3 µg/ml TBS containing 0.1% sodium azide and 1% NGS). Sections were then washed in TBS, incubated for 18–24 h with a 1 nm gold-conjugated goat-antirabbit IgG (H + L) serum

(British Biocell Int., Cardiff, Wales, UK) diluted 1:300 in TBS containing 0.1% sodium azide and 1% NGS. After thorough washing in TBS and then 0.1 M phosphate buffer (pH 7.4), sections were fixed for 10 min in 1% glutaraldehyde (in phosphate buffer), washed in deionised distilled H₂O (10× 5 min) and the immunoreaction was enhanced using a silver enhancing kit (British Biocell Int.). Sections were washed in deionised distilled H₂O, postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol followed by propylene oxide and embedded in Araldite. Approximately 12 ultrathin sections were cut from each Araldite-embedded specimen. These were stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope.

2.5. *P2X₇ receptor antibody and immunocytochemical controls*

The polyclonal antibody to P2X₇ was raised in New Zealand rabbits against specific peptide sequence of the P2X₇ receptor subtype and characterised by Roche Palo Alto, as described by Oglesby et al. (1999). This study showed that reabsorption of P2X₇ antibody with the homologous antigen (synthetic peptide used for the generation of the antibody) at a concentration of 5 µg/ml of diluted antibody (5 µg peptide: 3 µg antibody) was sufficient to abolish immunostaining. In the present study, the routine controls were applied with omission of the primary and secondary antibody steps, independently. These resulted in lack of immunolabelling.

2.6. *Controls to confirm that leucocyte infiltration is confined to oestrus*

Uterine tissue from non-oestrus controls was obtained (two rats in dioestrus and two in metoestrus) to confirm that there was no leucocyte infiltration into the endometrium during these stages.

2.7. *Isolation of rat spleen and uteri cells*

Eosinophil fractions were isolated based on the modified procedures of Wang et al. (2000) and Robertson et al. (2000). Briefly, spleen and uteri were collected from rats in oestrus. For splenic eosinophils, the spleens were collected and the eosinophils were gently removed by mechanical dissociation and re-suspended in buffered salt solution (BSS) and erythrocytes were lysed by a brief osmotic shock. The uteri were excised, trimmed of mesentery and fat, slit lengthwise, rinsed in PBS, then chopped into fine fragments and incubated in collagenase type I (1 mg/ml, Sigma Chemical Co., Poole, UK) plus DNAase 1 type II (25 µg/ml, Sigma) in DEMEM medium containing 10% fetal calf serum (FCS; 2.5 h at room temperature, with magnetic stirring). Tissue disruption was aided by repeated forceful pipetting at 30 min intervals during digestion. An equal volume of 5 mM EDTA in Hank's buffered salt solution (HBSS, without Ca²⁺ and Mg²⁺) was added 20 min before the end of the incubation. A single cell suspension was recovered by filtering the digestion mixture through a 70 µm cell strainer (Falcon, Franklin Lakes, NJ) to remove cells aggregate and tissue debris. After two washes in HBSS containing 5% FCS, on both preparations of cells (spleen and uteri) macrophages and lymphocytes were removed by centrifugation on a Ficoll density gradient (Amersham Pharmacia Biotech). Cells were further fractionated over a metrizamide gradient to enrich

the eosinophil population. Metrizamide was made up to 18.5 and 22.5% (w/v) solution in HBSS. Two milliliter of the 22.5% metrizamide solution were overlaid with 2 ml of the 18.5% solution in a 15 ml centrifuge tube. One milliliter of the 1×10^7 cells/ml suspension of non-adherent cells was added and the tube centrifuged for 15 min at $400 \times g$. Populations enriched with eosinophils were collected at the 18.5–22.5% metrizamide interface and kept on ice until use. Uteri from a total of nine rats in oestrus were used.

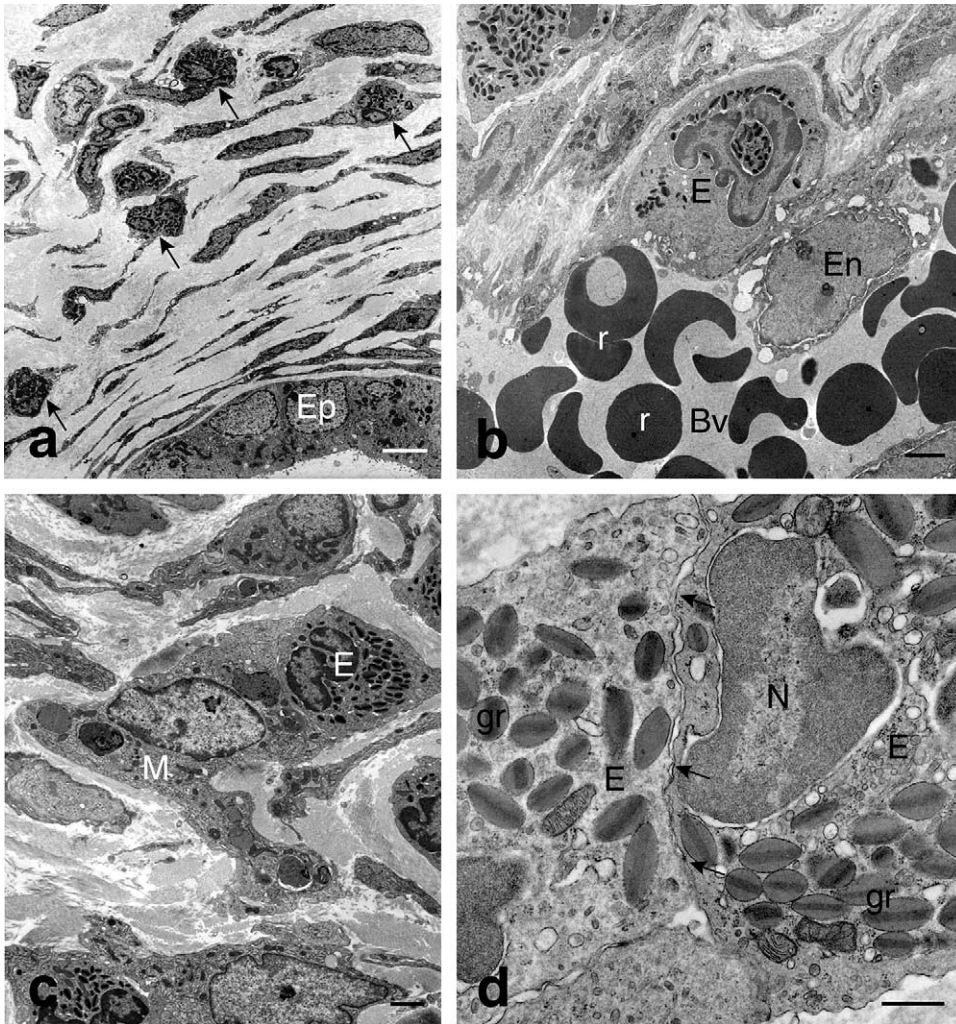


Fig. 1. Standard electron microscopy of uterine tissue during oestrus shows a diverse location of eosinophils. (a) A fragment of endometrial lamina propria showing numerous eosinophils (arrows). Ep—epithelium. (b) An eosinophil (E) associated with an endometrial blood vessel (Bv). En—endothelium; r—red blood cells. (c) Close relationship between an eosinophil (E) and a macrophage (M). (d) Higher magnification of contact between eosinophils (arrows). N—nucleus, gr—granules. Bar = $5 \mu\text{m}$ (a), $2 \mu\text{m}$ (b and c), 500 nm (d).

2.8. ATPe-induced permeabilization assay

To assess ATPe-induced membrane permeabilization, the membrane-impermeable DNA-staining fluorescent dye propidium iodide (PI) 2.5 μM (Molecular Probes, Eugene) was used. The spleen and uterus cell suspensions were pre-warmed for 5 min in PBS/bovine serum albumin at 37 °C and then incubated for 10 min either in the presence or absence of 5 mM ATP. During the last 5 min of incubation, the fluorescent dye PI (2.5 μM) was added to the cells. Samples of 5000 cells from three animals were acquired and the cells were gated by forward and side scatter for granulocytes, which were immediately analyzed using a FACScalibur (Becton & Dickinson, San Jose, CA, USA) flow cytometer. All data were analyzed using the WinMDI (Multiple Document Interface Flow Cytometry Application, V2.8) software.

2.9. 7-Amino actinomycin D (7AAD) staining and flow cytometry

In order to evaluate spontaneous apoptosis, freshly isolated spleen and uterus eosinophils cells, obtained from a total of nine animals were incubated for 40 min with 10 $\mu\text{g/ml}$ 7AAD (Sigma) at 4 °C in the dark. Samples of 10^4 cells stained with 7AAD and gated by forward and side scatter for granulocytes were analysed by FACScalibur (Becton & Dickinson) flow cytometer. Cells without 7AAD labeling were considered viable, while apoptotic and late-apoptotic/dead cells showed low and high 7AAD staining respectively (Hernandez et al., 2003; Philpott et al., 1996).

7AAD has the advantage over PI and related compounds in that it is able to identify early apoptotic cells (7AAD-dim), which retain membrane integrity separate from

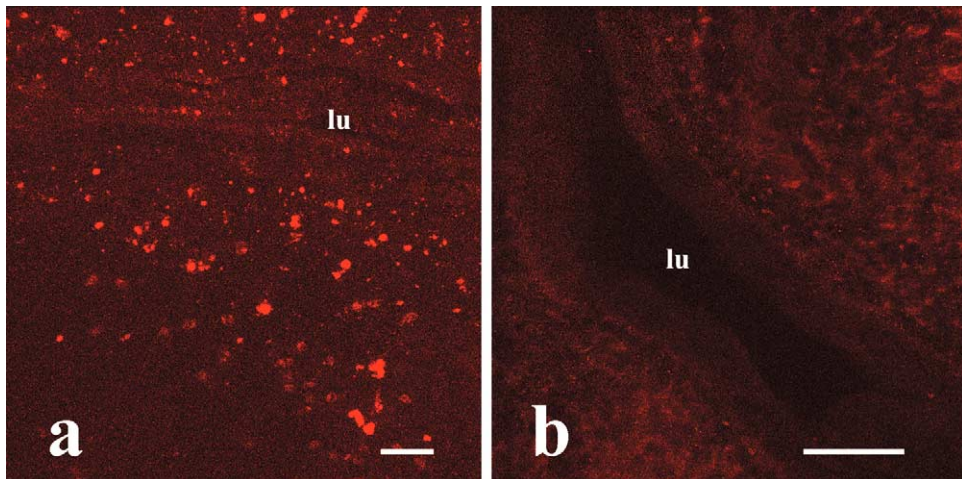


Fig. 2. Immunofluorescence-confocal microscopy of uterine tissue during oestrus. (a) The presence of P2X₇ receptor-positive cells (red labelled) in the lamina propria around the narrowed uterine lumen (lu). (b) An example of an immunocytochemical control preparation of uterus during oestrus showing no immunolabelling when anti-P2X₇ antibody step was omitted. Lumen, lu. Bar = 50 μm (a), 50 μm (b).

late-apoptotic/dead forms (7AAD-high), in which membrane integrity has been lost (Philpott et al., 1996).

3. Results

3.1. Electron microscopy

Standard electron microscopy confirmed the rich infiltration of eosinophils within the endometrium during oestrus (Fig. 1). These cells were easily recognized by the presence of characteristic cytoplasmic granules. Eosinophils were mostly located in the connective tissue of the lamina propria (Fig. 1a), where fibroblasts and macrophages could also be

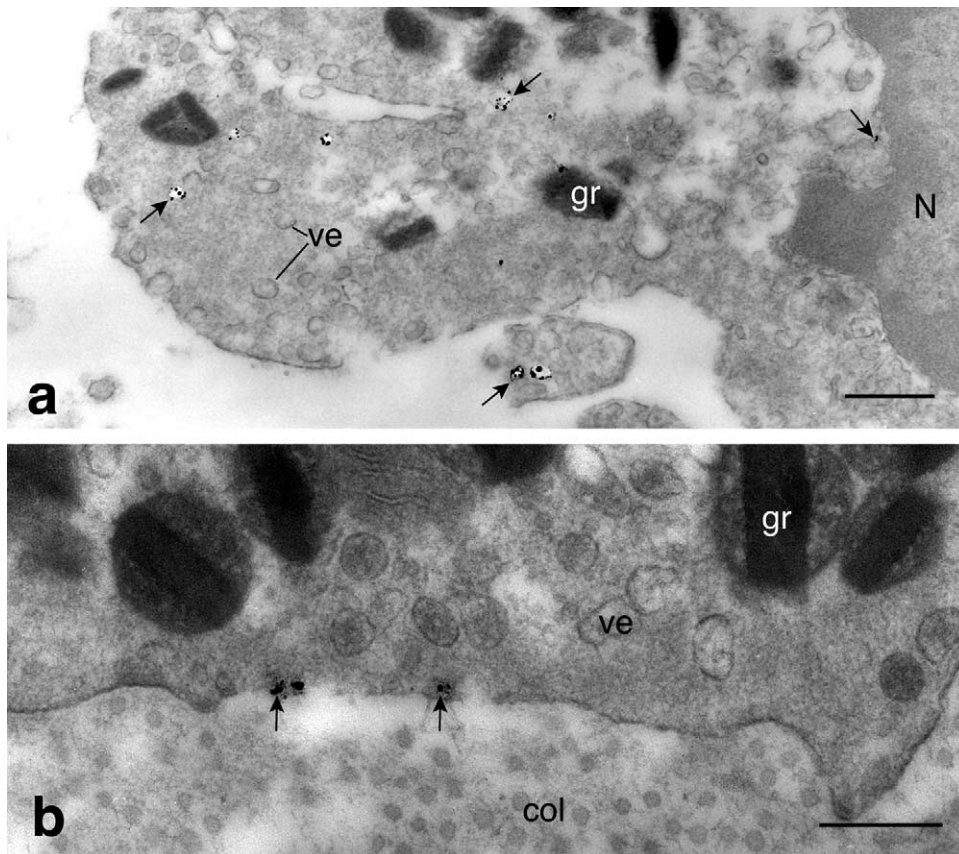


Fig. 3. Electron-immunocytochemistry of eosinophils labelled for P2X₇ receptors with immunogold–silver techniques. (a) Labelled eosinophils mostly show cytoplasmic and to some extent vesicle-associated distribution of black gold–silver grains (arrows). N—nucleus, gr—granules, ve—vesicles. (b) Higher magnification of labelled eosinophil displaying gold–silver grains in association with the cell membrane (arrows). ve—vesicle, gr—granules, col—collagen. Bar = 500 nm (a), 200 nm (b).

seen. It was often observed that eosinophils were associated with blood vessels (Fig. 1b) and macrophages (Fig. 1c). In addition, eosinophils were frequently clustered together and exhibited direct cell-to-cell contacts (Fig. 1d). No leucocyte infiltration was seen during dioestrus or metoestrus.

3.2. Immunofluorescence-confocal microscopy

P2X₇ receptor-positive immunoreactivity was observed in uterine tissues during oestrus (Fig. 2a). The immunoreactivity labelled a number of endometrial stromal cells. The intense

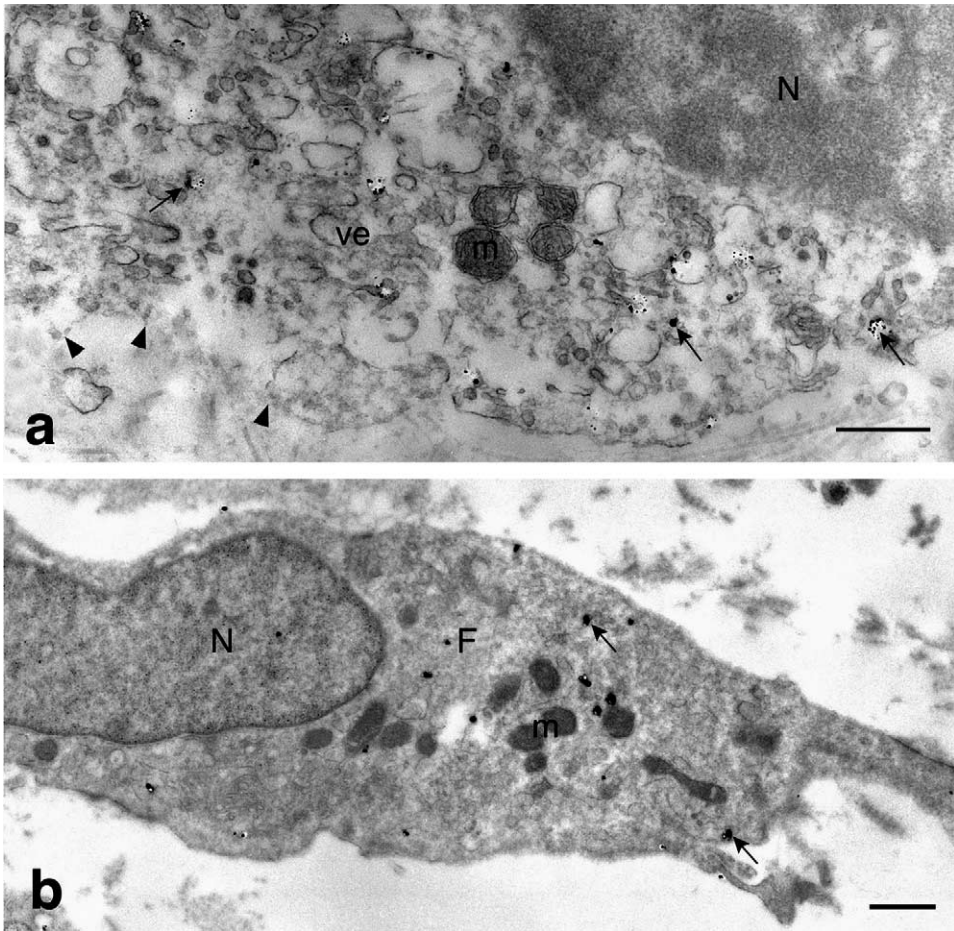


Fig. 4. Electron-immunocytochemistry of (a) macrophage and (b) fibroblast labelled for P2X₇ receptors with immunogold–silver techniques. (a) Macrophage displaying numerous gold–silver grains (arrows) in the cytoplasm; some grains are localised close to cellular organelles or structures including cytoplasmic vesicles. Note partial lysis of the cell membrane in the lower left region (arrowheads) suggesting cell apoptosis. N—nucleus, ve—vesicles, m—mitochondria. (b) Fibroblast (F) displays cytoplasmic location of immunoreactivity for P2X₇ receptors (arrows). N—nucleus, m—mitochondria. Bar = 500 nm (a and b).

labeling was observed in the cells located near the uterine lumen. In contrast, no immunoreactivity was observed in immunocytochemical control preparations of uterine tissue during oestrus (Fig. 2b).

3.3. Electron-immunocytochemistry

Immunogold–silver labeling techniques revealed immunoreactivity for P2X₇ receptors in the endometrial stromal cells of the rat uterus (Figs. 3 and 4). Labeling was observed in eosinophils (Fig. 3), macrophages and fibroblasts (Fig. 4). Gold–silver grains marking the presence of P2X₇ receptor proteins were noted in the cytoplasm, some clearly being vesicle-associated, and in association with the cell membrane, at least in some eosinophils (Fig. 3a

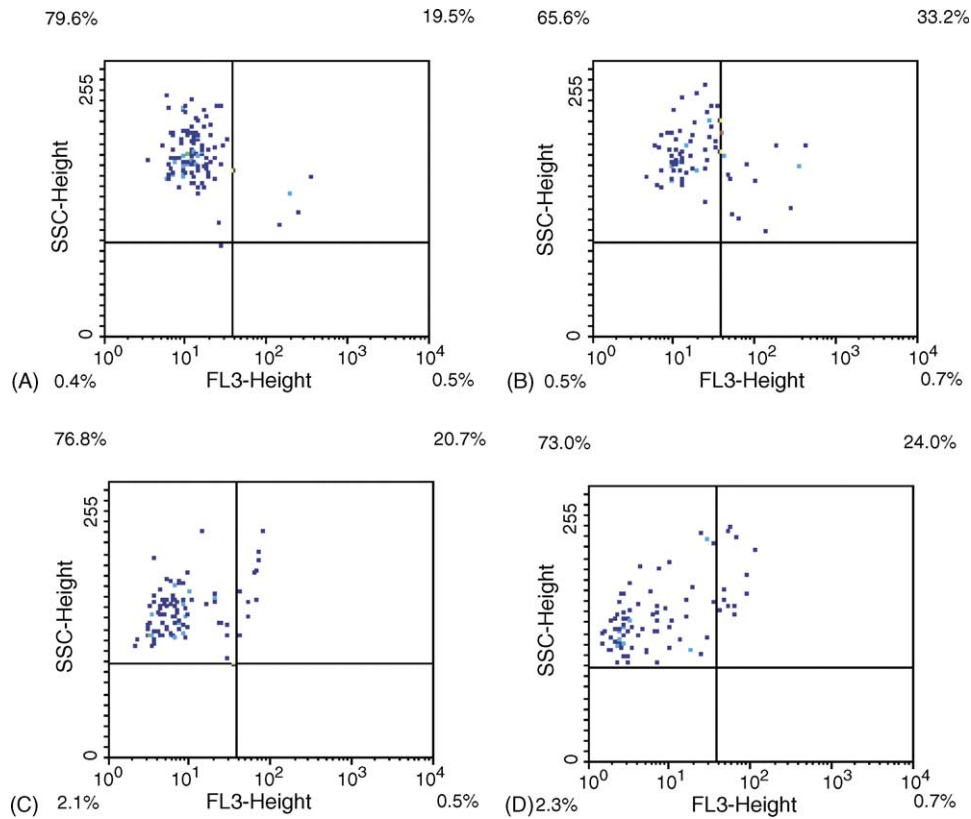


Fig. 5. ATP-induced permeabilization of uterine and spleen cells suspension. Eosinophils enriched cell population from uterus before (A) and after (B) ATP treatment. Eosinophils enriched cell population from spleen before (C) and after (D) ATP treatment. Note that in both cell groups ATP induced cell permeabilization, the effect on uterus cells was much greater than on cells of spleen origin. After isolation of the cells (as described in Section 2) the cells were selected for analysis based on forward and side scatter for granulocytes. Results are from a total of nine animals in oestrus, pooled tissue from three animals, repeated in triplicate.

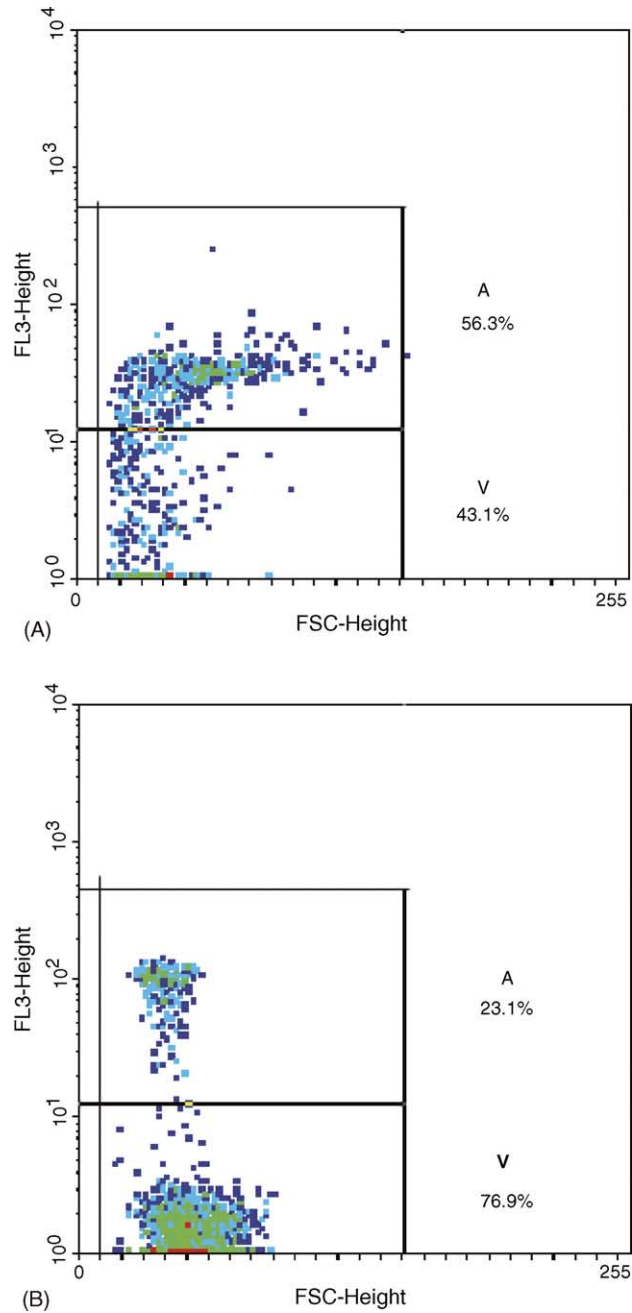


Fig. 6. Scattergrams of 7AAD-stained cells representing apoptotic (low staining) and late-apoptotic/dead (high staining) cells. (A) Cells isolated from uterus; (B) cells isolated from spleen. FSC, forward light scatter; FL3, 7AAD fluorescence; A, apoptotic; V, viable cells. Results are from a total of nine animals in oestrus, pooled tissue from three animals, repeated in triplicate.

and b). In labelled macrophages, gold–silver grains were mostly seen in the cytoplasm, some being vesicle associated, and to a lesser extent near the cell membrane (Fig. 4a). These cells usually contained numerous vesicles of various sizes and lipofuscin pigment granules. Fig. 4b is an example of the labelled fibroblasts, where gold–silver grains were mostly located in the cytoplasm.

3.4. ATPe-induced permeabilization on uterine and spleen cells

One of the main characteristics of functional P2X₇ expression in various cells types is the phenomenon of permeabilization. We assessed whether eosinophils from uterus and spleen were able to permeabilize their plasma membranes in the presence of ATP. As can be seen in Fig. 5, cells from both sites were sensitive to ATP but, while 33% of uterine cells became positively stained for PI after ATP treatment (Fig. 5A and B), only 24% of spleen cells were positively stained (Fig. 5C and D). If specific staining only is considered, then the difference between uterine and spleen cells is even greater—13 and 3% for uterine and spleen cells, respectively.

3.5. Apoptotic eosinophils from uterus and spleen

Using 7AAD staining to evaluate spontaneous apoptosis, we compared eosinophil staining from uterus and spleen. As shown in Fig. 6, there is more spontaneous apoptosis on cells isolated from uterus (Fig. 6A) than spleen (Fig. 6B). While approximately 56% of cells isolated from uterus were medium stained with 7AAD, only approximately 23% of cells isolated from spleen presented this medium staining.

4. Discussion

The well-known phenomenon of the rich presence of eosinophils in the rat endometrial stroma during oestrus with formation of eosinophil clusters in intimate cell-to-cell contact as described by Ross and Klebanoff (1966) was confirmed by electron microscopy in the present study. Furthermore, fluorescence microscopic examination showed that stromal cells displayed immunoreactivity for P2X₇ receptor protein during oestrus, whilst the application of electron-immunocytochemistry localised the immunoreactivity to eosinophils, macrophages and fibroblasts. These support the observations by Tassell et al. (2000), showing the presence of P2X₇ receptor protein in uterine stroma. Immunoreactivity for the P2X₇ receptor was primarily associated with the cell cytoplasm, in addition, some regions of the cell membrane and/or membrane-associated vesicles were clearly labelled. Intracellular localisation of the P2X₇ receptor is well documented (Bardini et al., 2000; Lee et al., 2000; Atkinson et al., 2002). This is characteristic of internalization and recycling of activated receptors as a part of the down-regulation process after in vivo stimulation (Dutton et al., 2000; Li et al., 2000; Ennion and Evans, 2001) or possibly due to a general intracellular receptor site for the P2X₇ receptors (Atkinson et al., 2002).

In preparation for receiving the fertilized ovum, many changes occur in the endometrium during oestrus. Endometrial leucocytes regulate these changes by their involvement in

uterine remodeling (Luque et al., 1997), cytokine secretion and development of immunity (Salamonsen et al., 2000). Our finding of the presence of P2X₇ receptor, a molecule associated with apoptosis, cytokine regulation and immune activities on endometrial leucocytes, suggests that many of the uterine functions regulated by leucocytes may be ATP-mediated.

ATP is known to mediate apoptosis by activation of P2X₇ receptors (Di Virgilio, 1995; Coutinho-Silva et al., 1999), and apoptotic epithelial cells of the skin and gut demonstrate strong immunoreactivity for the P2X₇ receptor (Gröschel-Stewart et al., 1999a,b). Similarly, apoptotic uterine epithelial cells adjacent to the implanting blastocyst have been found to be P2X₇ positive (Tassell et al., 2000) and, during oestrus, uterine epithelial cells express P2X₇ receptors (Bardini et al., 2000). In the present study, most of the eosinophils observed at the ultrastructural level did not show obvious morphological features of apoptosis. However, some of the P2X₇-positive macrophages displayed partial lysis of the cell membrane consistent with cell death. Also an increased number of apoptotic cells (eosinophils) in uterine stroma during oestrus were revealed using staining with 7AAD. Taken together, these findings support earlier observations by Tassell et al. (2000) suggesting that apoptotic processes take place within the rat uterus.

During early pregnancy, the embryo-fetus with its placental membranes is viewed as a successful allograft (Padykula and Campbell, 1976) and the ability of the uterine endometrium to deal with the conceptus is attributed to the sophistication of the cytokine network regulated by leucocytes (Salamonsen et al., 2000). Stromal cells secrete cytokines such as IL-1 and IL-6 needed for the implantation of the blastocyst (Salamonsen et al., 2000). Eosinophils do not constitutively express P2X₇, but human eosinophils were shown to do so when cultured with pro-inflammatory cytokines (Mohanty et al., 2001) and expression of P2X₇ receptors on eosinophils could be in itself an expression of activation. In accord with this hypothesis, we have observed that uterine cells are susceptible to extracellular ATP-induced permeabilization (a kind of fingerprint of P2X₇ receptor functional expression) (Di Virgilio et al., 2001).

Similarly, in fibroblasts and macrophages ATP stimulation via activation of P2X₇ receptors promotes maturation and release of the pro-inflammatory cytokines IL-1 β and IL-6 (Grahames et al., 1999; Solini et al., 1999). Although the exact role of the P2X₇ receptor on eosinophils, macrophages and fibroblasts in oestrus is not clear, it is reasonable to hypothesize that it plays an important role in uterine immune function by regulating ATP-mediated immune responses.

This study shows that the P2X₇ receptor is localised on eosinophils, macrophages and fibroblasts of the rat endometrium during oestrus. Some of the cells seem to undergo apoptosis, as revealed with 7AAD staining. P2X₇ receptor-positive cells have been observed in uterine tissue during oestrus at the electron microscopic level, allowing identification of positive cells by type. Findings suggest that the functions of eosinophils, as well as that of macrophages and fibroblasts, may be mediated through extracellular ATP acting via activation of the P2X₇ receptor. Further studies are required to determine the mechanism by which P2X₇ receptors modulate the function of these cells in the uterus during oestrus.

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