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Purinergic Signalling to Rat Ovarian Smooth Muscle: Changes in P2X Receptor Expression during Pregnancy

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Key Words

Immunohistochemistry · Ovarian smooth muscle · P2 receptor · Pregnancy · Rat (Sprague-Dawley)

Abstract

The expression of P2X and P2Y receptor subtypes in the smooth muscle of the rat ovary during the oestrus cycle and pregnancy was examined using immunohistochemistry. RT-PCR studies of P2X receptor mRNA were also carried out. In the non-pregnant rats, $P2X_2$ receptor protein was dominant in the smooth muscle of perifollicular rings and blood vessels. $P2X_1$ protein expression was seen on vascular smooth muscle too, but little, if any, was present on perifollicular smooth muscle. No changes in $P2X_1$ or $P2X_2$ receptor expression were seen during the oestrous cycle. During early and mid-late pregnancy, there was a switch from $P2X_2$ to $P2X_1$ receptor protein expression in the smooth muscle of the perifollicular ring; $P2X_1$ receptors were also more prominently expressed than $P2X_2$ receptors on ovarian vascular smooth muscle in non-pregnant animals, but during late pregnancy the expression of $P2X_2$ receptors was found to equal that of the $P2X_1$ receptors. There was a return to non-pregnant P2 receptor subtype distribution 2 days after birth. Ovarian vascular and perifollicular smooth muscle showed immunoreactivity for $P2Y_1$, but not for $P2X_{3–7}$, $P2Y_2$ or $P2Y_4$ receptors. $P2Y_1$ receptor expression in ovarian smooth muscle of both blood vessels and follicular rings did not show significant changes during the oestrus cycle or pregnancy. RT-PCR studies indicated that $P2X_1$ and $P2X_2$ receptor mRNA was present in the ovary during pregnant and non-pregnant conditions. $P2X_{4–6}$ receptor mRNA was also present in all stages studied, however no immunostaining showing receptor protein for these subtypes was seen on the ovarian sections examined. In summary, purinergic signalling to ovarian perifollicular smooth muscle changed from $P2X_2$ to $P2X_1$ receptors during pregnancy, while there was an increase in $P2X_2$ receptor expression on vascular smooth muscle.

Abbreviations used in this paper

ATP	adenosine 5′-triphosphate
NGS	normal goat serum
NHS	normal horse serum
PBS	phosphate-buffered saline

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Introduction

Several studies have indicated that adenosine 5′-triphosphate (ATP) is able to regulate ovarian function through binding to ATP receptors. For example, a P2 receptor was identified in bovine oviduct epithelium and it was postulated that this might influence the formation of oviduct fluid, which is necessary for gamete transport, as well as early development of the embryo [Cox and Leese, 1995]. P2 receptors have also been identified on human and porcine luteal and granulosa cells of the ovary [Soodak et al., 1988; Kamada et al., 1994]. ATP acting on P2Y receptors on human luteal cells [Lee et al., 1996; Squires et al., 1997] has been shown to increase the production of progesterone and oestradiol by these cells [Chen-Jei et al., 2000].

Recently, immunohistochemistry was used to describe the expression pattern of P2X receptors in the ovary during late pro-oestrus/early oestrus stage of the rat oestrous cycle [Bardini et al., 2000]. However, the role of ATP as a purinergic signalling molecule and the expression of the P2X receptor subtypes involved throughout the oestrous cycle and during pregnancy are still largely undefined, particularly with respect to smooth muscle within the ovary. The contraction of smooth muscle is crucial for many of the events that take place during the female reproductive cycle, such as the extrusion of the ovum from the ovary [Lipner and Maxwell, 1960; Amsterdam et al., 1977; Walles et al., 1978] and its subsequent conveyance via the oviduct to the uterus [Harper, 1988]. It was originally thought that visceral and vascular smooth muscle in general contained only $P2X_1$ receptors, but there is now evidence for the presence of $P2X_2$, $P2X_4$ and possibly $P2X_5$ receptors in some blood vessels [Nori et al., 1998; Hansen et al., 1999]. In the study of the non-pregnant female reproductive tract by Bardini et al. [2000], $P2X_1$ and $P2X_2$ receptor expression was immunolocalised to perifollicular and vascular smooth muscle within the ovary as well as the uterus.

Since there are marked differences in the control of ovarian function during pregnancy compared to during the oestrous cycle, it is hypothesised that this may be reflected by alterations in the expression of neurotransmitter receptors. There are hints that purinergic signalling may have important regulatory actions in the control of function of the ovary, and that ATP, acting via P2 receptors, may play a key role [Bardini et al., 2000]. Therefore, in this study, the expression of purine receptor subtypes within the rat ovarian smooth muscle during the oestrous cycle and pregnancy was the focus of interest. The study was carried out using polyclonal antibodies raised against $P2X_{1-7}$ and $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor peptides as well as RT-PCR studies of P2X receptor subtype mRNA. Co-localisation studies were also carried out to ascertain the distribution of smooth muscle and P2X receptors within the ovary. The possible functions and biological significance of the receptors expressed within ovarian smooth muscle are discussed.

Materials and Methods

Preparation of Samples

Animal experimentation was carried out in compliance with UK Home Office regulations [Animal (Scientific Procedures) Act 1986, Schedule 1]. Female Sprague-Dawley rats in prepubescence and the pro-oestrus/oestrus/metoestrus/dioestrus stage (as determined by vaginal smear and staining) were examined. For dated pregnancies, female Sprague-Dawley rats in oestrus were placed overnight with primed male rats and examined the following morning for the presence of a vaginal plug. The first day on which a plug was seen was designated gestation day 1. Three stages of pregnancy were examined (early pregnancy – day 5, mid-pregnancy – day 12 and late pregnancy – day 22) as well as 1 and 2 days after pregnancy. Four rats for each experimental stage were examined. The rats were killed by asphyxiation with a rising concentration of carbon dioxide and cervical dislocation (as appropriate under Schedule 1 of the Animal Act, 1986), and both ovaries were then removed. The tissue was embedded in OCT compound (BDH/Merck, Leicester, UK) and frozen in isopentane, which had been precooled in liquid nitrogen. The tissues were sectioned at 12 μm using a cryostat (Reichert Jung CM1800), collected on gelatin-coated slides and air-dried at room temperature. The slides were stored at –20°C and allowed to return to room temperature for at least 15 min prior to use.

Immunohistochemistry

The immunogens used for the production of polyclonal antibodies (made available from Roche Bioscience, Palo Alto, Calif., USA) were synthetic peptides corresponding to 15 receptor-type-specific amino acids in the carboxy termini of the cloned rat P2X receptors. The peptide sequences are as follows:

$P2X_1$ amino acids 385–399 ATSSTLGLQENMRTS
$P2X_2$ amino acids 458–472 QQDSTSTDPKGLAQL
$P2X_3$ amino acids 383–397 VEKQSTDSGAYSIGH
$P2X_4$ amino acids 374–388 YVEDYEQGLSGEMNQ
$P2X_5$ amino acids 437–451 RENAIVNVKQSQILH
$P2X_6$ amino acids 357–371 EAGFYWRTKYEEARA
$P2X_7$ amino acids 555–569 TWRFVSQDMADFAIL

The peptides were linked to keyhole limpet haemocyanin, and the conjugate was administered to New Zealand rabbits at monthly intervals to raise the polyclonal antibodies. IgG fractions were isolated from the immune and pre-immune sera ($P2X_{1-7}$). The specificity of the antibodies was verified by immunoblotting with membrane preparations from CHO-K1 cells expressing the cloned $P2X_{1-7}$ receptors (all steps above were performed by Research Genetics, Huntsville, Ala., USA).

Table 1. P2X receptor primers for RT-PCR on total RNA extracted from rat ovarian tissue

Primer	Position	Sequence (5′ to 3′)	Annealing temperature, °C	Predicted length, bp
$P2X_1$	776–801 (S) 1,203–1,231 (AS)	GAAGTGTGATCTGGACTGGCACGT GCGTCAAGTCCGGATCTCGACTAA	58	452
$P2X_2$	826–845 (S) 1,183–1,164 (AS)	GAATCAGAGTGCAACCCCAA TCACAGGCCATCTACTTGAG	61	357
$P2X_3$	708–731 (S) 1,126–1,147 (AS)	TGGCGTTCTGGGTATTAAGATCGG CAGTGGCCTGGTCACTGGCGA	58	440
$P2X_4$	749–774 (S) 1,170–1,195 (AS)	GAGGCATCATGGGTATCCAGATAAG GAGCGGGGTGGAAATGTAACTTTAG	58	447
$P2X_5$	553–577 (S) 944–970 (AS)	GCCGAAAGCTTCACCATTTCCATAA CCTACGGCATCCGCTTTGATGTGATAG	58	418
$P2X_6$	444–468 (S) 938–963 (AS)	AAAGACTGGTCAGTGTGTGGCGTTC TGCCTGCCCAGTGACAAGAATGTCAA	57	520
$P2X_7$	384–410 (S) 711–737 (AS)	GTGCCATTCTGACCAGGGTTGTATAAA GCCACCTCTGTAAAGTTCTCTCCGATT	58	354

The peptides corresponding to amino acids in the carboxy termini of the P2Y receptors (available from Alomone Laboratories, Jerusalem, Israel) have the following sequences:

$P2Y_1$ amino acids 242–258 RALIYKDLDNSPLRRKS

$P2Y_2$ amino acids 227–244 KPAYGTTGLPRAKRKSVR

$P2Y_4$ amino acids 337–350 HEESISRWADTHQD

For immunostaining of cryostat sections, the avidin-biotin complex technique was used based on the protocol developed by Llewellyn-Smith et al. [1992, 1993]. Air-dried sections of the tissues were fixed for 2 min in 4% formaldehyde plus a 0.2% solution of saturated picric acid in 0.1 *M* sodium phosphate buffer (pH 7.4). After washing in phosphate-buffered saline (PBS) for 15 min, endogenous peroxidase activity was blocked by treating the sections with 0.4% hydrogen peroxide and 50% methanol for 10 min. Three 2-min PBS washes were then carried out. Following this, non-specific binding sites were blocked by a 20-min incubation in 10% normal horse serum (NHS) in PBS containing 0.05% Merthiolate (Sigma, Poole, UK). The P2X antibodies were then diluted to 1:400 with 10% NHS-PBS Merthiolate (10% NHS plus 0.2% Triton was used in the case of $P2X_3$) and the P2Y antibodies were diluted to 1:200 ($P2Y_1$ and $P2Y_2$) and 1:100 ($P2Y_4$). The sections were then incubated with the primary P2 antibody overnight.

The following day, after three 5-min washes in PBS, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) diluted 1:500 in 1% NHS-PBS Merthiolate for 1 h. A further three 5-min washes in PBS were carried out and then the sections were incubated for 1 h at room temperature in ExtrAvidin peroxidase (Sigma) diluted 1:1,500 in PBS-Merthiolate. Three 5-min washes in PBS were then carried out. For a colour reaction, a solution containing 0.05% 3,3-diaminobenzidine, 0.04% nickel ammonium sulphate, 0.2% β-*D*-glucose, 0.004% ammonium chloride and 1 U/ml glucose oxidase in 0.2 *M* sodium phosphate buffer (pH 7.4) was applied to the sections for 6 min. The specimens were dehydrated to xylene and mounted in Eukitt (BDH/Merck). Control experiments were carried out by omission of the primary antibody and by pre-absorbtion of the primary antibody with the corresponding peptide used to immunise the rabbits.

Sections were visualised using a Zeiss Axioplan microscope (Jena, Germany), and images were captured using a Leica DC200 digital camera.

Immunofluorescence Co-Localisation with α-Smooth Muscle Actin and Anti-$P2X_1$ and Anti-$P2X_2$ Antibodies

Air-dried sections of tissue were fixed for 2 min in 4% formaldehyde in 0.1 *M* sodium phosphate buffer (pH 7.4). After washing in PBS for 15 min, sections were then treated with a 20-min 10% normal goat serum (NGS) incubation to block non-specific binding sites. Sections were then incubated overnight at room temperature in 2.5 μg/ml of either rabbit $P2X_1$ or $P2X_2$ antibody diluted with 10% NGS. After this incubation, all washes were carried out using PBS containing 0.05% Tween 20 (Sigma). The sections were washed and $P2X_1$ or $P2X_2$ receptor expression visualised by incubation for 1 h with Oregon-green-labelled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratory) diluted 1:100 with NGS. Next, a further 15-min wash was carried out. Double-labelling of smooth muscle cells was performed by overnight incubation with mouse anti-smooth muscle actin antibody (Sigma) diluted 1:1,000 with 10% NGS. The next day sections were washed and smooth muscle actin expression was visualised by incubation for 1 h with TRITC-labelled goat anti-mouse antibody (Jackson ImmunoResearch Laboratory) diluted 1:100 with 10% NGS. The sections were washed in PBS and mounted immediately in Citifluor. Care was taken throughout the immunofluorescence studies to ensure the sections were exposed to as little light as possible to prevent bleaching of the fluorescent markers. The sections were visualised using a Zeiss Axioplan microscope (Jena, Germany) and images were captured using a Leica DC200 digital camera.

Table 2. Results of the immunohistochemical studies showing the expression of $P2X_1$, $P2X_2$ and $P2Y_1$ receptors within smooth muscle structures of the ovary at different stages of the oestrous cycle and pregnancy

	$P2X_1$		$P2X_2$		$P2Y_1$	
	vascular smooth muscle	perifollicular smooth muscle	vascular smooth muscle	perifollicular smooth muscle	vascular smooth muscle	perifollicular smooth muscle
Pre-pubescent	++		+	+	++	++
Pro-oestrus	++		+	++	++	++
Oestrus	++		+	++	++	++
Metoestrus	++		+	++	++	++
Dioestrus	++		+	++	++	++
Early pregnancy	++	++	+		++	++
Mid-pregnancy	++	++	+		++	++
Late pregnancy	++	++	++		++	++
Post-pregnancy day 1	++	(+)	+	(+)	++	++
Post-pregnancy day 2	++		+	++	++	++

The scores reported were based on blinded observation of treatments. Treatments showing no score indicates that staining was not detected in the ovarian sections analysed. Four rats per investigated stage of the oestrous cycle and pregnancy were utilized, and both ovaries from each animal were examined. ++ = Strong immunostaining; + = weak immunostaining; (+) = very weak immunostaining.

RT-PCR

Total RNA was extracted from rat ovarian tissue at the stages of the oestrus cycle and pregnancy described previously, using the SV Total RNA Isolation System (Promega, Southampton, UK). 1 μg of total RNA was used for RT-PCR. Reverse transcription and cDNA amplification for all the P2X receptors were carried out with a thermal cycler (Hybaid, Basingstoke, UK) in a one-step protocol using Ready-to-Go RT-PCR Beads (Amersham Pharmacia Biotech, Little Chalfont, UK). Primer sequences (Life Technologies, Paisley, UK) for $P2X_{1-7}$ were used for the amplification reactions, as summarised in table 1 [Shibuya et al., 1999].

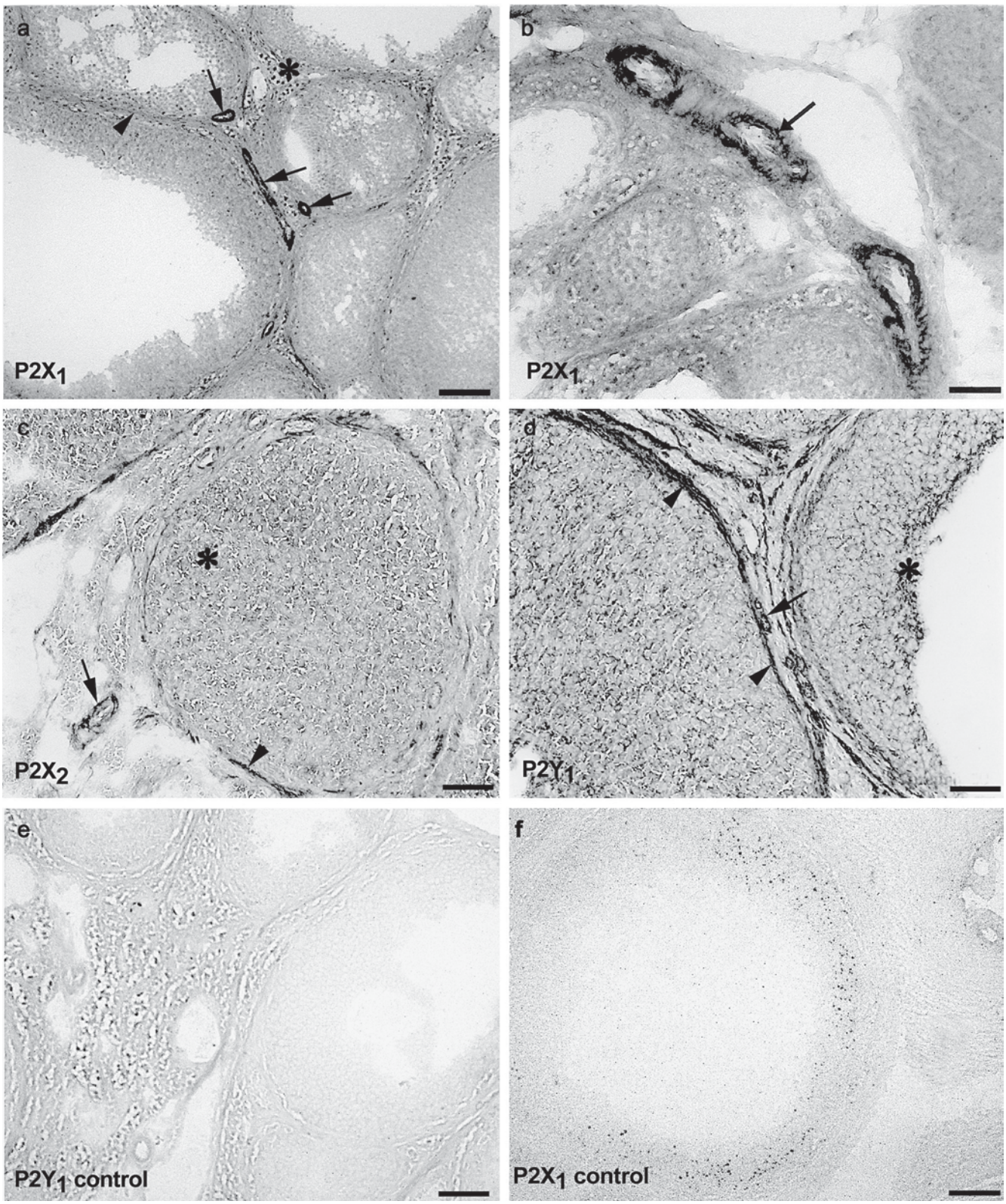
The amplification reaction, performed in the same reaction tube, was conducted under the following conditions: 95°C for 30 s, the relevant annealing temperature for 1 min, and 72°C for 1 min, plus an additional cycle with an elongation time of 5 min. Amplification products were separated by electrophoresis and visualised by ethidium bromide staining. The presence of possible contaminants was investigated using control RT-PCR reactions in which either mRNA had been omitted or the reverse transcriptase had been inactivated by heating to 95°C.

Results

A summary of the immunohistochemical studies showing the expression of $P2X_1$, $P2X_2$ and $P2Y_1$ receptors during pre-pubescence, different stages of the oestrous cycle and pregnancy, and post-pregnancy day 1 and day 2 are given in table 2 and presented sequentially in figures 1–6. No immunostaining for $P2X_{3-7}$, $P2Y_2$ or $P2Y_4$ was seen on the ovarian smooth muscle in all the stages studied. Incubations omitting the primary antibody and pre-absorption controls were used to establish the level of non-specific background staining. Pre-absorption of the P2X antibody and the P2Y antibody with excess of the corresponding peptide used for immunisation showed a small amount of background reactivity (fig. 1e for $P2Y_1$; fig. 1f for $P2X_1$; fig. 4e for $P2X_2$).

Fig. 1. Pre-pubescence. **a**, **b** $P2X_1$ immunoreactivity was seen only on the vascular smooth muscle (arrow) and not on the perifollicular smooth muscle though some degree of background staining is present (arrowhead). There was also $P2X_1$ immunoreactivity seen within the ovarian stroma (*). **a** Scale bar = 100 μm. **b** Scale bar = 25 μm. **c** $P2X_2$ immunoreactivity was seen within the blood vessel walls (arrow) and on the perifollicular ring (arrowhead). The positive band of staining around the follicle was thin and discontinuous. $P2X_2$ immunoreactivity was also seen on granulosa cells (*). Scale bar = 100 μm. **d** $P2Y_1$ immunoreactivity was localised on the perifollicular ring (arrowhead) and the vascular smooth muscle (arrow). $P2Y_1$ immunostaining was also seen on the granulosa cell layer surrounding the follicular antrum (*). Scale bar = 100 μm. **e** Pre-absorption of the $P2Y_1$ antibody with its cognate peptide gave no immunostaining on smooth muscle, but some background staining is present. Scale bar = 100 μm. **f** Pre-absorption of the $P2X_1$ antibody with its cognate peptide gave no immunostaining for vascular smooth muscle, but some background staining was revealed. Scale bar = 100 μm.

Pre-pubescence



Oestrous cycle

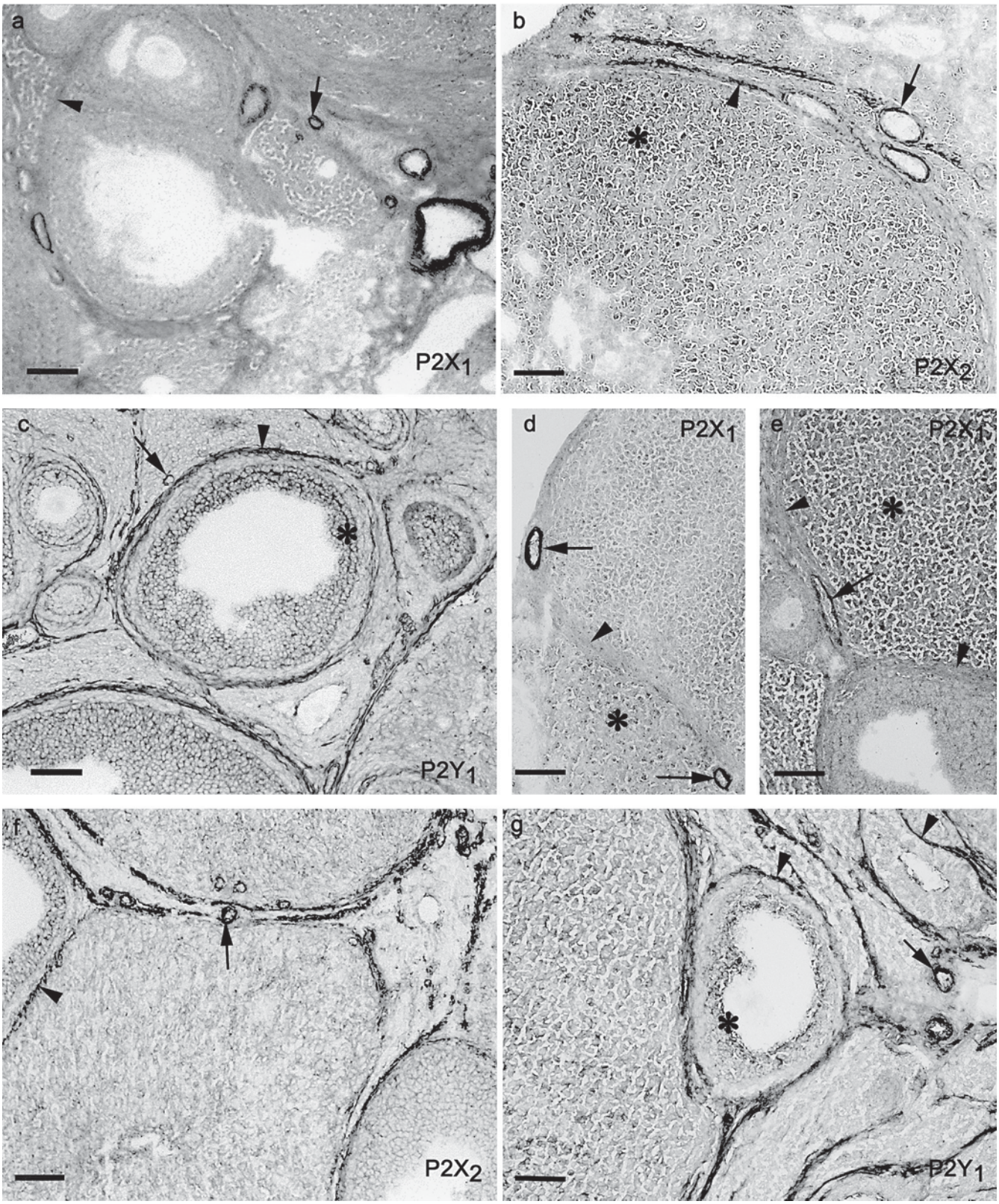


Figure 7 shows immunofluorescence co-localisation of smooth muscle actin and $P2X_2$ receptor distribution within the ovary during oestrus.

P2 Receptor Expression during Pre-Pubescence and the Oestrous Cycle

During pre-pubescence and during the investigated stages of the oestrous cycle, immunoreactivity of the blood vessel walls to $P2X_1$ was observed but there was no reaction of the perifollicular layer with the antibody to $P2X_1$ (fig. 1a for pre-pubescence; fig. 2a for pro-oestrus; fig. 2d for oestrus, and fig. 2e for dioestrus). $P2X_2$ receptors, during pre-pubescence and all the stages of the oestrous cycle, were immunolocalised on small and large blood vessels, though immunoreactivity was less than that seen to $P2X_1$ on these structures (fig. 1c for pre-pubescence; fig. 2b for pro-estrus). $P2X_2$ was also immunolocalised on perifollicular theca externa cells (fig. 1c, 2b). These results are consistent with those obtained for the late pro-oestrus/early oestrus stage investigated previously by Bardini et al. [2000].

$P2X_1$ and $P2X_2$ immunoreactivity was also occasionally seen in the ovarian stroma of some of the sections examined (fig. 1a). Immunofluorescence studies of smooth muscle actin and P2X receptors suggest that immunopositive $P2X_1$ and $P2X_2$ cells which appear in the perifollicular theca externa, vascular structures and ovarian stroma are co-localised with smooth muscle actin. Figure 7 shows an example of $P2X_2$ and α-smooth muscle actin co-localisation.

$P2Y_1$ receptors were shown to be immunolocalised on vascular and perifollicular smooth muscle (fig. 1d, 2c, f) as well as on the cells of the granulosa layer of follicles within the ovary (fig. 1d, 2c, f).

P2 Receptor Expression during Pregnancy

During early, mid-, and late pregnancy, immunostaining of $P2X_1$ receptors was seen on smooth muscle cells of blood vessel walls, but in contrast to the non-pregnant ovary, $P2X_1$ receptors were also immunolocalised on the perifollicular smooth muscle ring (e.g. fig. 3a, b, 4a, b, 5a, b). $P2X_2$ receptors, on the other hand, were seen to be localised only on vascular smooth muscle and immunoreactivity was present very weakly, if at all, in perifollicular smooth muscle cells (fig. 3c, 4c, d, 5c).

During early and mid-pregnancy, $P2X_1$ receptor immunostaining of blood vessels was predominant and was seen in medium-large vessels that were in close association with follicles (fig. 3a, b, 4a, b). $P2X_2$ receptor staining of blood vessels at this stage was present, but the degree of immunoreactivity to $P2X_2$ appeared to be less than that seen for $P2X_1$ (fig. 3c, 4c, d). The ovarian tissue during pregnancy appeared to become more vascular compared to during the oestrous cycle, and there were indications of hypertrophy of blood vessels, particularly during mid-pregnancy (fig. 4b). The blood vessels seen during late pregnancy showed immunoreactivity to $P2X_1$ receptors (fig. 5a, b) and $P2X_2$ receptors (fig. 5c) and, in contrast to mid-pregnancy, $P2X_1$ receptor immunostaining of vascular smooth muscle was found to equal that of $P2X_2$.

During pregnancy, $P2Y_1$ receptor immunoreactivity was again shown to be localised on vascular and perifollicular smooth muscle (fig. 3d, 4f, 5d).

P2 Receptor Expression after Pregnancy

After pregnancy, the pattern of $P2X_1$ and $P2X_2$ receptor staining began to resemble that seen during the oestrous cycle. One day after pregnancy, immunoreactivity to $P2X_1$ receptors was seen in vascular smooth muscle, but appeared only weakly in perifollicular cells (fig. 6a). By post-pregnancy day 2, immunoreactivity of $P2X_1$ receptors was seen in blood vessel walls but not in the perifollicular ring (fig. 6b). $P2X_2$ immunostaining, though only weak 1 day after pregnancy (fig. 6c), was also immu-

Fig. 2. Oestrous cycle. **a** Pro-oestrus: with antibody to $P2X_1$ only blood vessels immunostained (arrow). The perifollicular ring of developing follicles showed no immunostaining to $P2X_1$ (arrowhead). Scale bar = 100 μm. **b** Pro-oestrus: $P2X_2$ was immunolocalised to the perifollicular ring (arrowhead) and the walls of blood vessels (arrow). $P2X_2$ immunoreactivity was also seen on granulosa cells (*), perhaps representing receptor clusters on the cell surface. Scale bar = 100 μm. **c** Pro-oestrus: $P2Y_1$ immunostained perifollicular smooth muscle (arrowhead) and vascular smooth muscle (arrow). $P2Y_1$ immunostaining was also seen on the granulosa cell layer. Scale bar = 100 μm. **d** Oestrus: With antibody to $P2X_1$ only blood vessels immunostained (arrow). Immunoreactivity to $P2X_1$ was not seen within the developing perifollicular layer (arrowhead). $P2X_1$ immunoreactivity was also seen on granulosa cells (*). Scale bar = 100 μm. **e** Dioestrus: $P2X_1$ was immunolocalised only on the walls of blood vessels (arrow). The perifollicular ring of follicles showed no immunoreactivity to $P2X_1$ (arrowhead). $P2X_1$ immunoreactivity was also seen on granulosa cells (*). Scale bar = 100 μm. **f** Oestrus: $P2X_2$ was immunolocalised to the perifollicular ring of developing follicles (arrowhead) and the blood vessel walls (arrow). Scale bar = 100 μm. **g** Oestrus: $P2Y_1$ immunostained both the vascular smooth muscle (arrow) and the perifollicular smooth muscle (arrowhead). $P2Y_1$ immunostaining was also seen on the granulosa cell layer (*). Scale bar = 100 μm.

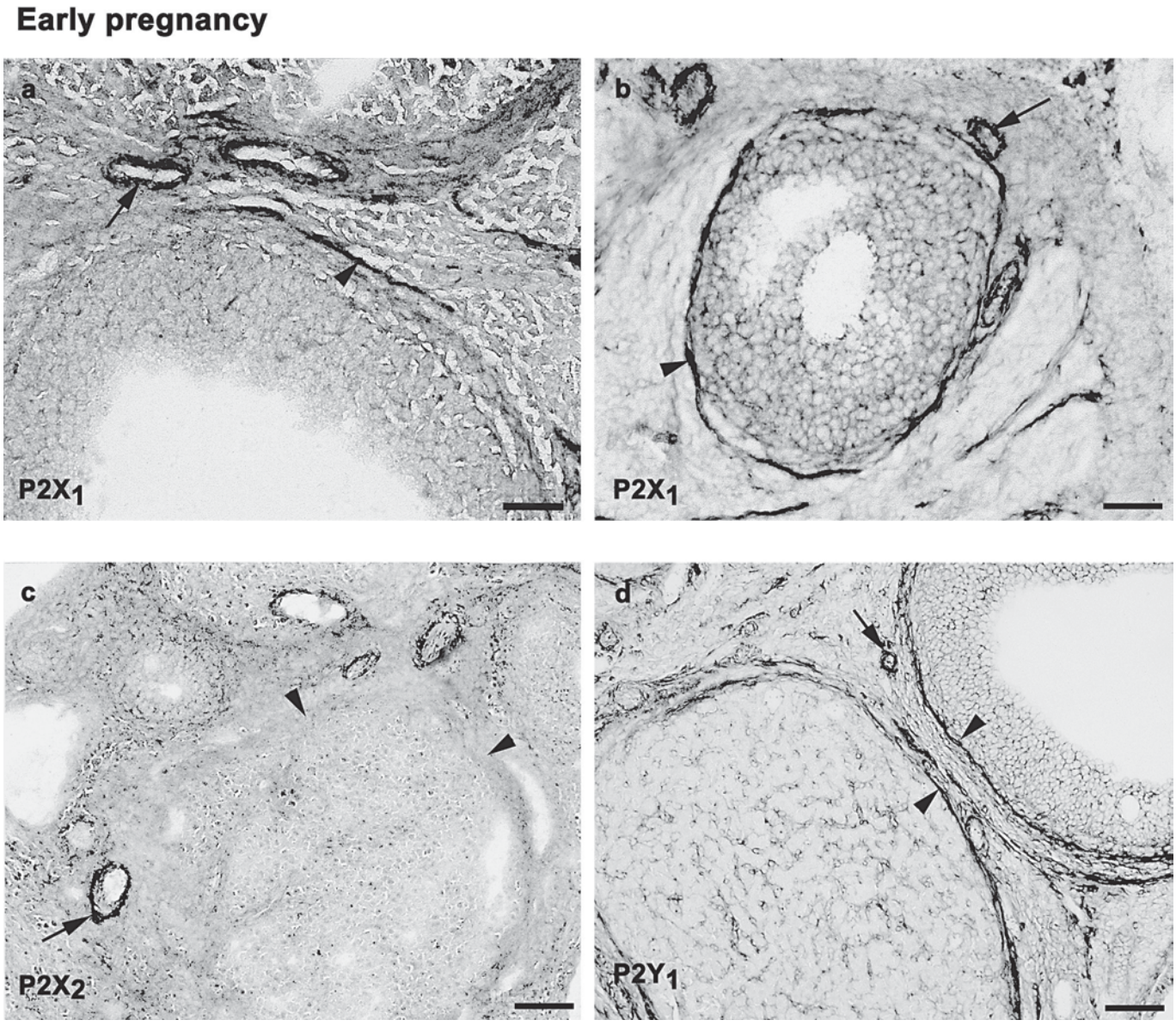


Fig. 3. Early pregnancy. **a**, **b** Immunoreactivity to $P2X_1$ antibody was seen in the vascular smooth muscle (arrow) and perifollicular smooth muscle (arrowhead). **a** Scale bar = 50 μm. **b** Scale bar = 100 μm. **c** $P2X_2$ was immunolocalised to vascular smooth muscle (arrow) but not to the perifollicular ring (arrowhead). Scale bar = 100 μm. **d** $P2Y_1$ was immunolocalised to the perifollicular smooth muscle (arrowhead) and the vascular smooth muscle (arrow). Scale bar = 100 μm.

nolocalised in the perifollicular ring and was more visible 2 days after pregnancy (fig. 6d), reminiscent of the pattern of staining seen during the oestrous cycle.

With respect to the blood vessels, $P2X_1$ and $P2X_2$ both immunostained vascular smooth muscle at the post-pregnancy stage (fig. 6a–d). The pattern of immunostaining for P2X receptor subtypes of vascular smooth muscle during post-pregnancy was similar to that seen during the oestrous cycle where $P2X_1$ receptors were predominant.

The immunostaining pattern of $P2Y_1$ receptors was shown to be localised in vascular and perifollicular smooth muscle (fig. 6e, f). The granulosa cells showed immunoreactivity to $P2Y_1$ receptors but this was weaker than that seen during late pregnancy, and appeared to be confined

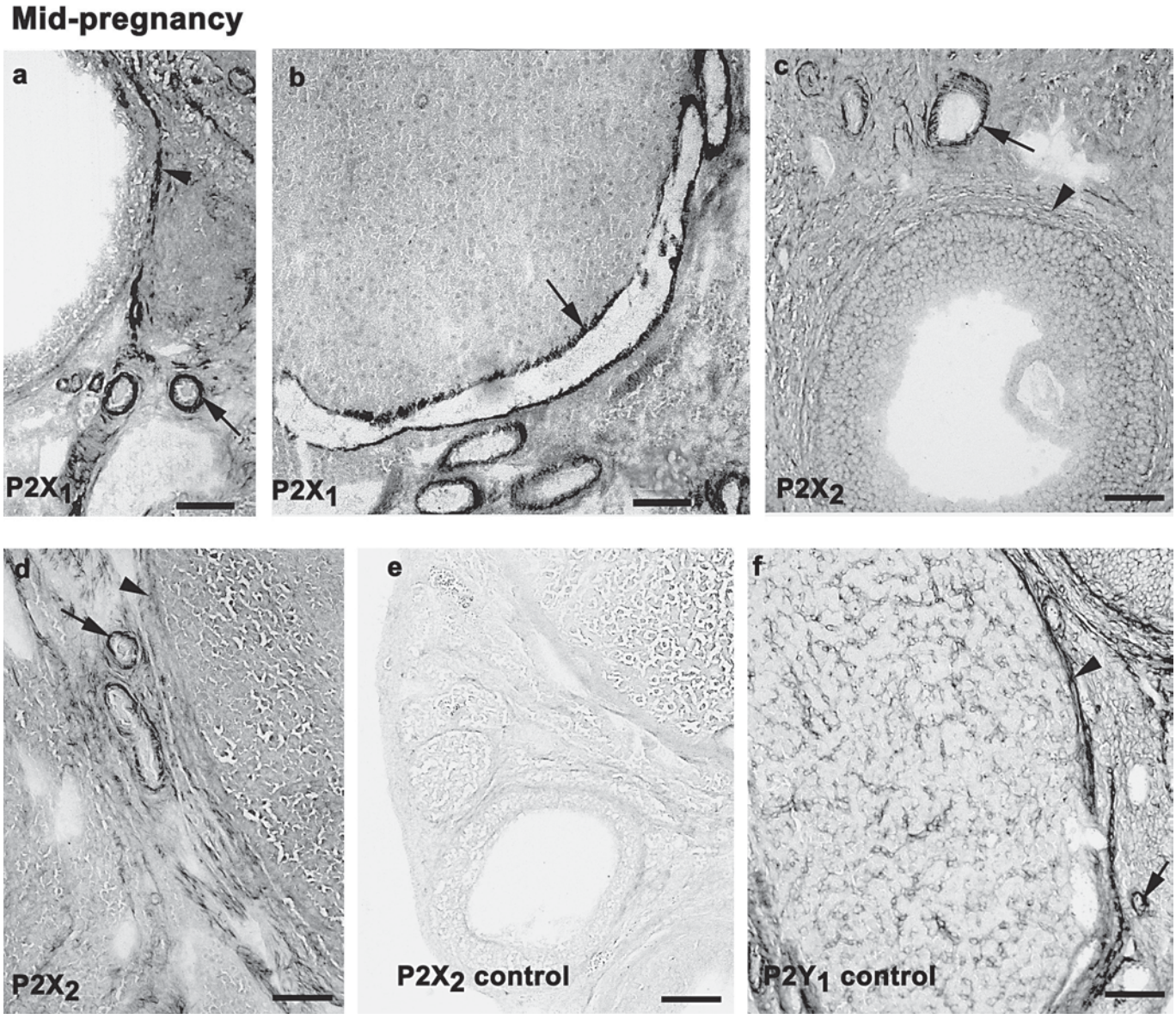


Fig. 4. Mid-pregnancy. **a**, **b** immunostaining with $P2X_1$ was seen in the perifollicular ring (arrowhead) and the blood vessels (arrow). Scale bar = 100 μm. **c**, **d** $P2X_2$ was immunolocalised to blood vessels (arrow). The perifollicular ring of follicles showed no staining to $P2X_2$ (arrowhead). Scale bar = 100 μm. **e** Preabsorption of the $P2X_2$ antibody with its cognate peptide gave no staining of smooth muscle, but revealed some background staining. Scale bar = 100 μm. **f** $P2Y_1$ was immunolocalised to blood vessel walls (arrow) and the perifollicular ring (arrowhead). Scale bar = 100 μm.

to the granulosa cells surrounding the antrum of the follicle (fig. 6f).

RT-PCR Studies

RT-PCR studies showed that mRNA for $P2X_{1,2,4,5,6}$ was present in rat ovarian tissue during all the investigated non-pregnant and pregnant stages (see fig. 8 for results from metoestrus). Though $P2X_{4-6}$ mRNA was detected by RT-PCR, there was no immunostaining of these receptors in the ovarian sections examined suggesting post-transcriptional expression of these receptors did not occur.

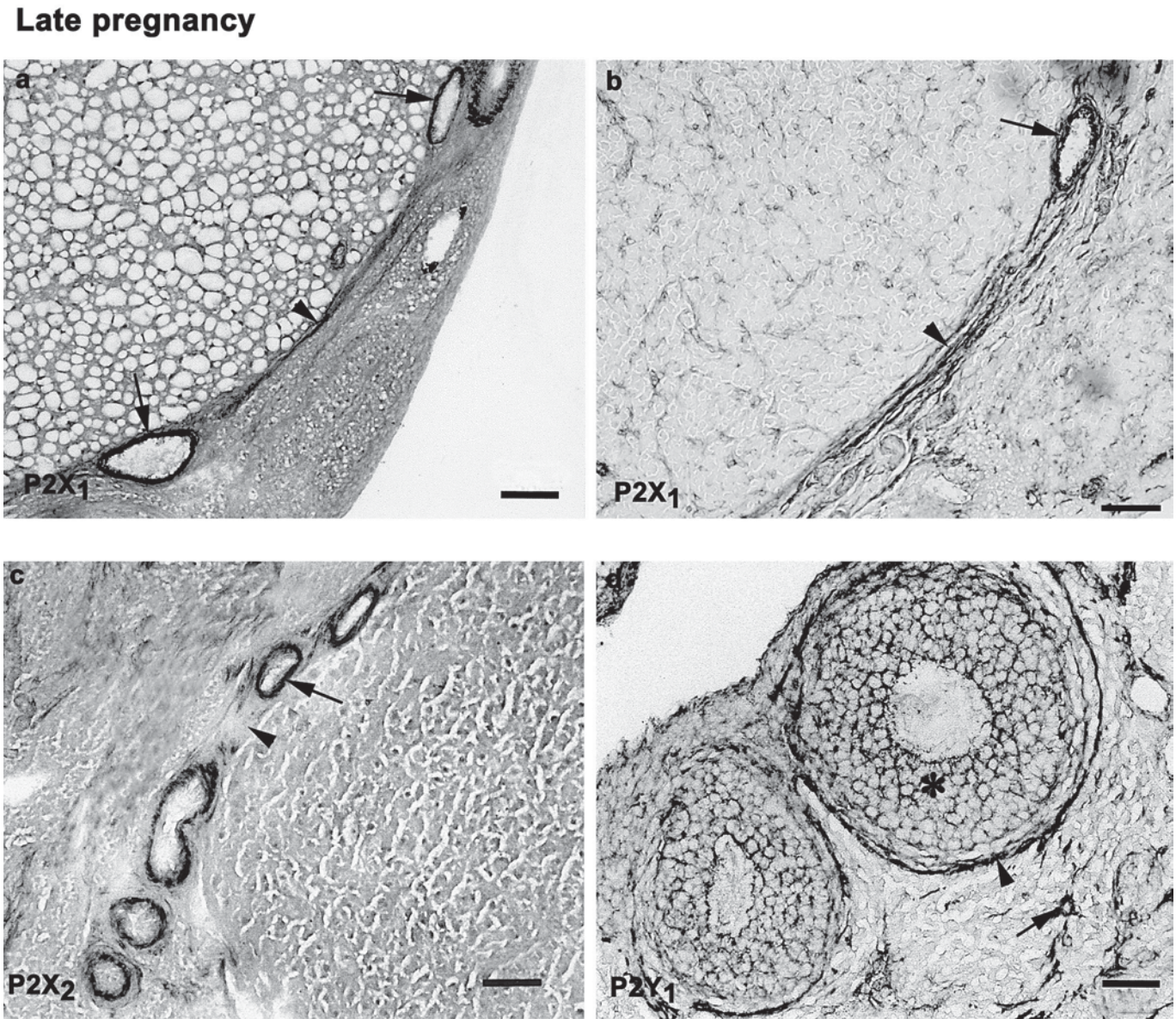
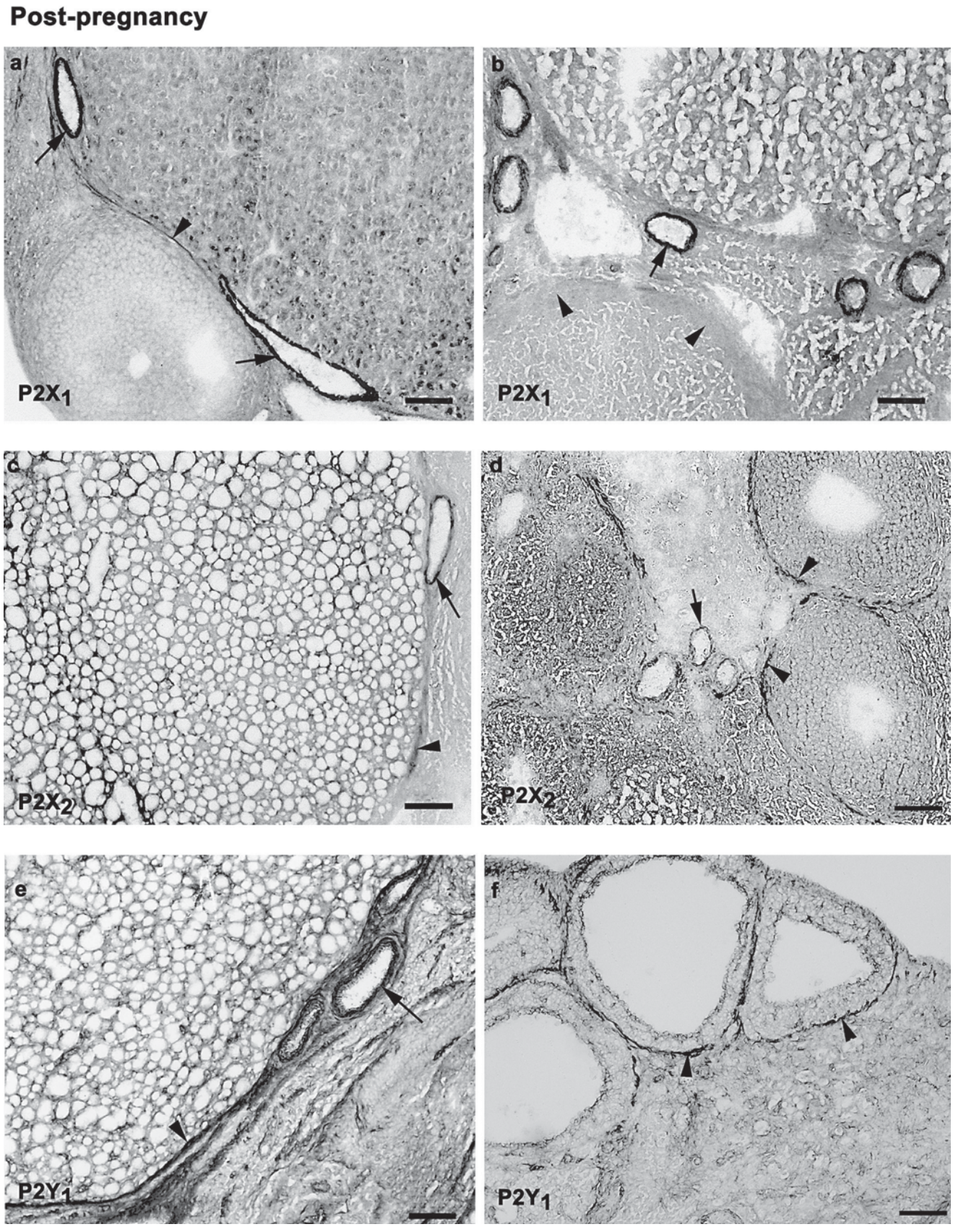


Fig. 5. Late pregnancy. **a**, **b** $P2X_1$ was immunolocalised to the perifollicular ring of mature follicles (arrowhead) and the vascular smooth muscle (arrow). The large follicle indicated by the arrowhead may have been arrested during development due to pregnancy halting ovulation. Scale bar = 100 μm. **c** $P2X_2$ immunostaining was seen in the walls of blood vessels (arrow) but not on the perifollicular ring (arrowhead). Scale bar = 100 μm. **d** $P2Y_1$ immunostaining was seen in the perifollicular ring (arrowhead) and on the walls of blood vessels (arrow). $P2Y_1$ immunostaining was also seen on the granulosa cell layer (*). Scale bar = 50 μm.

Fig. 6. Post-pregnancy. **a** Post-pregnancy day 1: $P2X_1$ immunostaining of blood vessels (arrow) and weak $P2X_1$ immunostaining on the perifollicular ring of mature follicles (arrowhead) was seen. Scale bar = 100 μm. **b** Post-pregnancy day 2: $P2X_1$ immunostaining of blood vessels (arrow) was seen, but the perifollicular ring of mature follicles showed no immunoreactivity to $P2X_1$. Scale bar = 100 μm. **c** Post-pregnancy day 1: $P2X_2$ immunostaining of blood vessels (arrow) and weak $P2X_2$ immunostaining on the perifollicular ring of mature follicles (arrowhead) was seen. Scale bar = 100 μm. **d** Post-pregnancy day 2: $P2X_2$ immunoreactivity was seen in blood vessels (arrow) and the perifollicular ring of maturing follicles (arrowhead). Scale bar = 100 μm. **e**, **f** Post-pregnancy day 2: $P2Y_1$ immunostained blood vessels (arrow) and the perifollicular ring (arrowhead). Scale bar = 100 μm.



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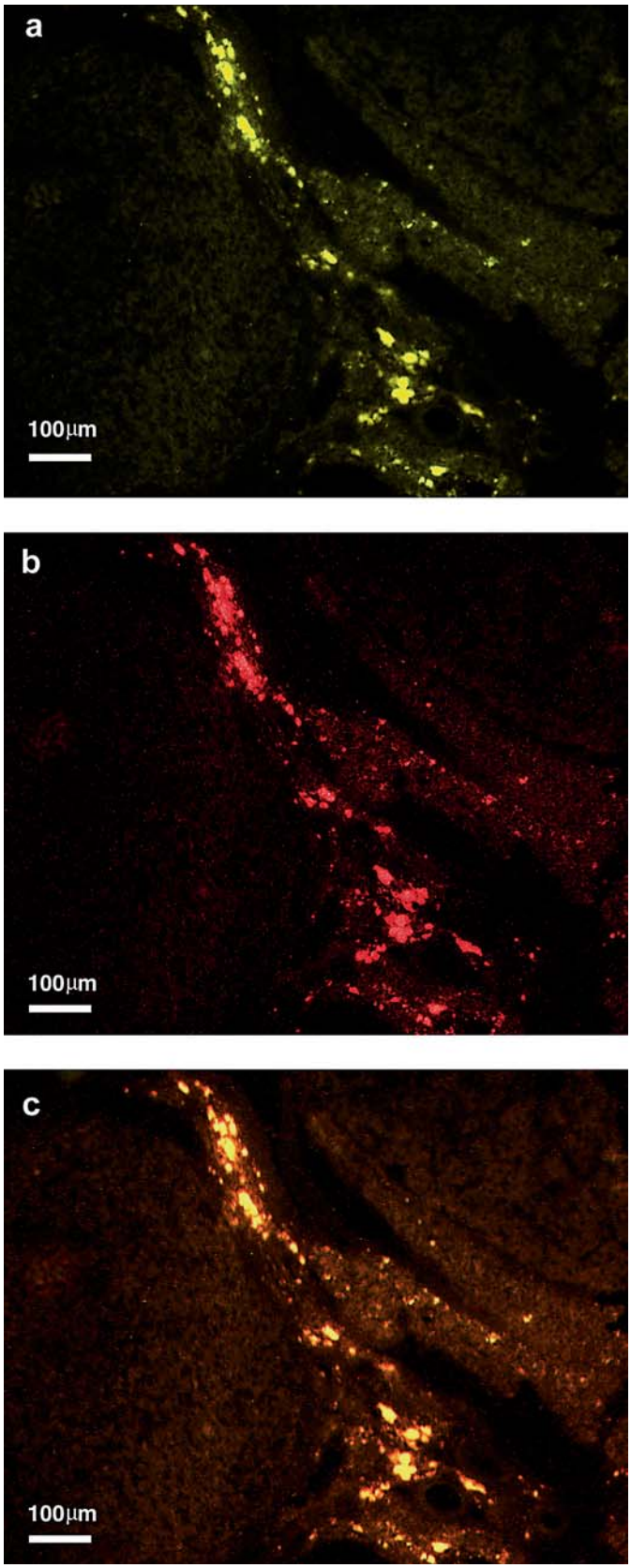

Discussion

Perifollicular Smooth Muscle

The most striking change that occurred during the oestrous cycle and pregnancy was the switch from $P2X_2$ to $P2X_1$ receptors in the smooth muscle cells of the perifollicular ring. During pre-pubescence, there was a small amount of immunoreactivity to $P2X_2$, which stained for a thin, discontinuous, undeveloped smooth muscle layer (theca externa) around the developing follicles. During the oestrous cycle, $P2X_2$ receptors were strongly expressed in the perifollicular smooth muscle theca externa layer. Immunoreactivity for $P2X_1$ receptors was rarely observed in the perifollicular smooth muscle during pre-pubescence and during the cycle.

Electron microscopy has demonstrated cells with typical smooth muscle myofilaments in the theca externa of large follicles in the rat as well as in the ovarian stroma [O'Shea, 1970; Osvaldo-Decima, 1970]. A study by Amsterdam et al. [1977] examined the distribution of actin and myosin in the rat ovary and revealed several layers of cells within the theca externa, forming a consistent band around the larger follicles and an incomplete band around the smaller follicles. During follicular growth, it has been suggested that contraction of the theca externa layer in response to catecholamines may play a role in the extrusion of the oocyte at ovulation [Jacobowitz and Wallach, 1969]. It is postulated that during the oestrus cycle, ATP released as a cotransmitter with noradrenaline from sympathetic nerves [Burnstock, 1990], acts on P2X receptors on smooth muscle cells of the perifollicular ring to elicit contraction of the theca externa prior to ovulation.

It is possible that the $P2X_2$ receptor is better adapted to fulfil the ultimate goal of ovulation and hence it dominates in the perifollicular layer during the oestrous cycle. The pattern of immunostaining of the perifollicular theca externa with P2X receptor subtypes changed during pregnancy (early, mid- and late pregnancy). The perifollicular

Fig. 7. Immunofluorescence co-localisation of $P2X_2$ receptor expression and smooth muscle actin during oestrus. **a** $P2X_2$-positive perifollicular and ovarian stromal cells stained green with Oregon green. **b** Smooth muscle actin-positive cells stained red with TRITC. **c** Co-localisation of $P2X_2$ with smooth muscle actin within the perifollicular ring and ovarian stroma.

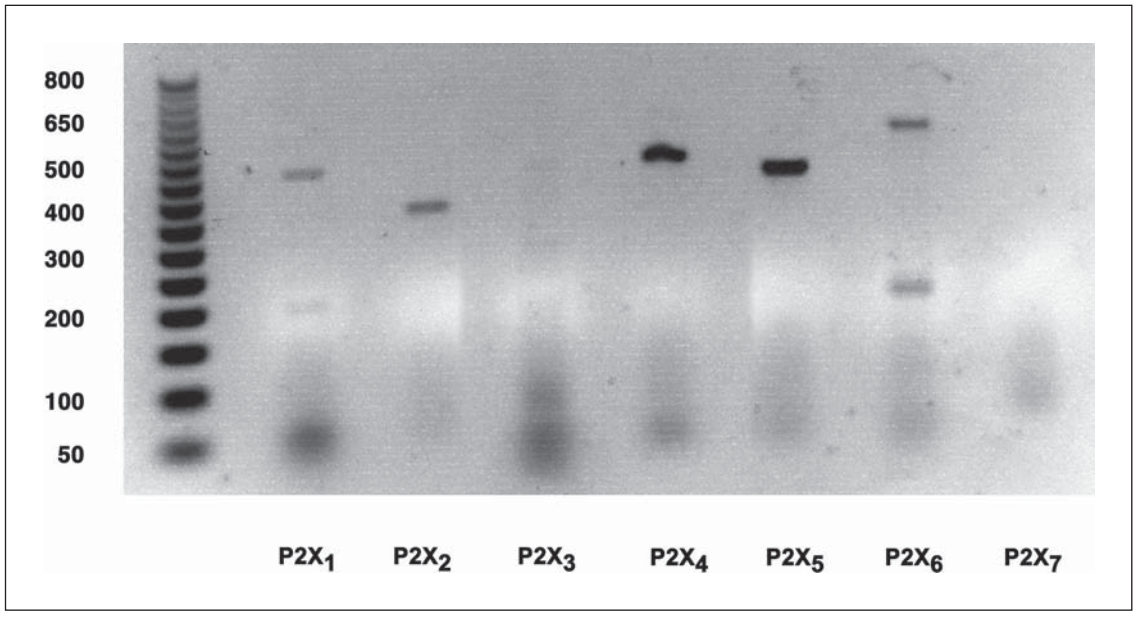

Fig. 8. RT-PCR analysis of P2X purinoceptor mRNA expressed in the rat ovary during metoestrus: mRNA for $P2X_{1,2,4,5,6}$ was present as shown by a clear-cut band.

smooth muscle during pregnancy was seen to show predominantly $P2X_1$- rather than $P2X_2$-positive staining, in contrast to the pattern of staining seen during the oestrous cycle. Extrusion of the oocyte is not seen during pregnancy and this may account for the fact that $P2X_2$ receptor expression in the perifollicular smooth muscle is absent in this condition.

In a study of purinoceptor signalling in smooth muscle phenotypes from the aorta, plasticity of receptor expression was demonstrated [Erlinge et al., 1998]. $P2X_1$ was prominent in the contractile phenotype, but in the synthetic phenotype grown in culture, the $P2X_1$ receptor was not detectable, while the $P2Y_1$ and $P2Y_2$ receptors were substantially upregulated. In the present study, RT-PCR results showed that receptor mRNA for both $P2X_1$ and $P2X_2$ subtypes were present in both the pregnant and non-pregnant ovary. However there is a switch of smooth muscle receptor subtype expression from $P2X_2$ during the oestrous cycle to $P2X_1$ during pregnancy. The two receptors have different properties. a-β-methylene ATP is a potent agonist of the $P2X_1$ receptor, which is rapidly desensitised [Burnstock, 2001]. The $P2X_2$ receptor lacks fast desensitisation [Ralevic and Burnstock, 1998] and is particularly sensitive to acidity and Zn^{2+} [King et al., 1996; Wildman et al., 1998]. During the oestrous cycle, when follicles are constantly developing and undergoing atresia, inflammatory reactions would presumably occur leading to alterations in pH. It may be that the $P2X_2$ receptor, which is sensitive to changes in acidity, is better suited as a neurotransmitter receptor in this situation. During pregnancy, when the development of follicles is temporarily suspended, the $P2X_1$ receptor, which is desensitised rapidly, may be better adapted to not only cope with the demands of an increased blood supply, but also to prevent the contraction of the theca externa and hence the release of ova from the follicles.

One day after pregnancy, the perifollicular ring stained for $P2X_1$ and weakly for $P2X_2$. By post-pregnancy day 2 the pattern of staining resembled that seen during the oestrous cycle, with $P2X_2$-positive smooth muscle cells in the theca externa dominating. $P2X_1$ immunoreactivity in the perifollicular ring was not seen. In the rat, as in several other animals, ovulation occurs shortly after parturition [Long and Evans, 1922]. This quick recurrence of the follicular development and ovulation following parturition may explain the rapid return to the non-pregnant expression of the $P2X_2$ purinoceptor subtype.

Vascular Smooth Muscle

Sympathetic, parasympathetic and sensory nerves innervate the blood vessels of the rat ovary [Adashi and Leung, 1993]. The vasoconstrictor tone of these vessels is

likely to be controlled by the sympathetic nerves that, in other sites, have been shown to release both noradrenaline and ATP, which act on α-adrenergic and P2X receptors, respectively [Burnstock, 1990]. $P2Y_1$ receptors are present on endothelial cells and have been shown to mediate vasodilation [Ralevic and Burnstock, 1998].

During pre-pubescence, cohorts of follicles develop and then degenerate without ovulation occurring. A complex vascular network is formed within the thecal cell layer during follicular growth [Otani et al., 1999], and in this study, immunoreactivity of those blood vessels in close association with follicles was seen for $P2X_1$ and $P2X_2$, with $P2X_1$ receptor expression appearing to predominate.

During the oestrous cycle, $P2X_1$ and $P2X_2$ were both expressed in blood vessels throughout the stroma and in close association with follicles, and again $P2X_1$ immunostaining was predominant.

Immunostaining of blood vessels with $P2X_1$ and $P2X_2$ was seen during early, mid- and late pregnancy. During mid-pregnancy, the medium-large blood vessels in close association with follicles showed strong immunoreactivity with $P2X_1$ antibody compared to $P2X_2$. In general, there was evidence of increased vascularisation during pregnancy compared to that seen during the oestrous cycle. The increasing vascularisation that is continued from the latter stages of early pregnancy throughout mid-pregnancy may in part be to supply the developing corpora lutea of pregnancy. These enlarge from day 10 (mid-pregnancy) onwards [Mossman and Duke, 1973] and also bring about degeneration of all the other corpora from previous ovulations [Long and Evans, 1922].

The fact that vessels in close association with the follicles are predominantly immunoreactive to $P2X_1$ could again be related to the fact that $P2X_1$ receptors rapidly desensitise. This may be better suited to give a short-lasting constrictor response to ATP under the conditions of follicular development in the oestrous cycle and early and mid-pregnancy when vasodilation, leading to an increased blood vascular supply, is desired.

Smaller blood vessels than those seen during early and mid-pregnancy were seen in late pregnancy and these showed immunoreactivity to $P2X_1$ and $P2X_2$. The smaller size of the vessels and the fact that immunoreactivity to $P2X_1$ does not seem to predominate may reflect the fact that the corpora no longer need such a controlled blood supply because by this time the placenta is firmly established and has taken over the endocrine role, and furthermore, at this time the corpora of pregnancy usually begin to degenerate.

$P2Y_1$ Receptor Expression

An unexpected finding of this study was the association of G-protein-coupled $P2Y_1$ receptors with perifollicular and vascular smooth muscle. The expression pattern of this receptor subtype appears to remain constant throughout the oestrous cycle and pregnancy. The $P2Y_1$ receptor has been shown to mediate vasodilatation in the smooth muscle of rabbit portal vein, and coronary and mesenteric arteries [Ralevic and Burnstock, 1998]. Within the ovary this receptor may help to mediate vasodilation, as well as mediate contraction or relaxation of perifollicular smooth muscle. Perhaps the nature of its role is influenced by which other P2 receptor subtypes are expressed within the same structure.

It has been suggested that ATP and adenosine 5′-diphosphate acting on P2Y receptors may regulate proliferation of smooth muscle cells after injury [Burnstock, 2002]. The long-term trophic role within the ovary of the $P2Y_1$ receptor could therefore also be considered. For example, extrusion of an oocyte from its follicle would clearly result in damage to the perifollicular layer and perhaps the $P2Y_1$ receptors present help to promote proliferation as the empty follicle undergoes the transformation into the corpus luteum. The $P2Y_1$ receptors present in vascular smooth muscle may be present to promote cell proliferation and hypertrophy of the blood vessels during certain times, such as prior to oestrus or during pregnancy.

In conclusion, the presence of P2X receptors and P2Y receptors in ovarian structures may reflect a significant role for ATP involved in purinergic signalling in the ovary. The timing of receptor expression seems to be closely related to key events involving follicular development in the oestrus cycle and during pregnancy. Hormonal influences during pregnancy have previously been shown to dramatically alter the expression and distribution of P2X subtypes in the pregnant rat bladder [Yunaev et al., 1999]. It may be that activation of purine receptors initiates a number of critical changes that facilitate ovulation and the maintenance of pregnancy.

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