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REGULAR ARTICLE

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Changes in expression of P2 receptors in rat and mouse pancreas during development and ageing

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Abstract In view of the evidence for a role for extracellular ATP in both pancreatic endocrine and exocrine functions, we have investigated the expression of P2X and P2Y receptors in this tissue in neonate and aged rat and mouse. Using immunohistochemistry it was shown that $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors were present in different regions of the rat and mouse pancreas; $P2X_3$ and $P2X_6$ receptors were not found, and $P2X_5$ immunolabelling was only found in some nerves. The pancreatic vasculature of both rat and mouse expressed $P2X_1$, $P2X_2$, $P2Y_1$ and $P2Y_2$ receptors in the smooth muscle. $P2X_1$ and $P2X_4$ receptors were absent in the islets of the neonate pancreas, but were progressively upregulated with age after birth. In contrast, the greatest expression of $P2Y_1$ in cells from the duct system was in neonate pancreas, while there was no $P2Y_1$ expression in aged rat pancreas. $P2X_7$ receptors had a consistent pattern of distribution in all of the groups examined, being located in the outer periphery of the islet. Using antibodies raised against insulin, somatostatin and glucagon, double-labelling immunofluorescence was used to identify $P2X_7$-positive cells in different islet of Langerhans cell populations. Our results demonstrated a clear immunoreaction to $P2X_7$ receptors in islet α cells, while no $P2X_7$ was expressed in β and δ cells. The significance of the differential expression of P2 receptors in the pancreas during development and ageing, and a possible role for the proliferation and death of the islet cell population are discussed.

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Introduction

The pancreas is an organ with both endocrine and exocrine functions. The pancreatic duct and acinar cells secrete most of the fluid and determine the final electrolyte composition of the pancreatic juice, while the acinar cells also secrete digestive enzymes. The islets are responsible for endocrine function, with secretion mainly of glucagon by α cells, insulin by β cells, somatostatin by δ cells and pancreatic polypeptide by γ cells (Junqueira et al. 1998).

It has long been known that exogenous adenosine tri- and diphosphate nucleotides have effects on functions of the pancreas (Candela and Garcia-Fernandes 1963; Levine et al. 1970; Loubatières-Mariani et al. 1979; Chapal and Loubatières-Mariani 1981). Initially it was shown that exogenous adenosine and nucleotides interfere with insulin secretion. Whereas adenosine inhibits insulin secretion, ATP stimulates it. Later, extracellular nucleotides were shown to control vascular tone, probably following their release from sympathetic nerves (Chapal and Loubatières-Mariani 1983; Hillaire-Buys et al. 1998). Exogenous ATP may elicit both vasoconstriction (via activation of a P2X receptor on vascular smooth muscle) and vasodilatation (via P2Y receptors on the endothelium). Extracellular nucleotides may also exert effects on pancreatic duct cells (Chan et al. 1996; Christoffersen et al. 1998; Luo et al. 1999).

Nucleotides are known to affect many cellular processes via P2 receptors that have been subdivided into P2X and P2Y families (Ralevic and Burnstock 1998). The P2X receptors are ionotropic, ligand-gated cation channels, and P2Y receptors are G protein-coupled receptors. Up to now seven members of the P2X ($P2X_{1–7}$) family have been cloned and six subtypes of mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and

$P2Y_{12}$; Jacobson et al. 2000; Hollopeter et al. 2001). Both P2X and P2Y receptor subtypes have been described in pancreatic tissues, mainly from pharmacological studies. A “pancreas-specific human putative P2 receptor” was cloned in 1996 (Stam et al. 1996) that was later identified as the $P2Y_4$ receptor that had been cloned in the previous year by Communi et al. (1995; see Ralevic and Burnstock 1998). Recently, based on measurement of $[Ca^{2+}]$ in microperfused intralobular ducts and RT-PCR analysis, it has been proposed that $P2X_1$, $P2X_4$, $P2X_7$ and $P2Y_1$ receptors are present on the basolateral membrane and $P2Y_2$, $P2Y_4$, and $P2X_7$ (and possibly $P2Y_5$) receptors on the luminal membrane of pancreatic duct cells (Luo et al. 1999).

In the present study, we investigated, using immunohistochemistry, the expression of the seven subtypes of the P2X receptor family, and $P2Y_1$ and $P2Y_2$ receptors in the pancreas of neonate, mature and aged rat and in mature mouse.

Materials and methods

Animals

Breeding, maintenance and killing of the animals used in this study followed the principles of good laboratory animal care and experimentation in compliance with the UK national law and regulations. Pancreatic tissues were taken from three groups of male Sprague-Dawley rats (1-day-old and 3-day-old neonates; 16-week-old mature adults; and 24-month-old aged adults) and from 8- to 10-week-old mature adult male Balb c and Swiss mice. Animals were kept in a constant 12 h/12 h light-dark cycle with free access to food and water. The animals were killed by exposure to an increasing dose of carbon dioxide, and death was confirmed by cervical dislocation.

Immunohistochemistry

Tissue handling

The pancreas was removed, put in HBSS solution, embedded in OCT tissue compound (BDH), progressively frozen in isopentane (pre-cooled in liquid nitrogen) and then stored in liquid nitrogen. Cryostat sections were cut as sets of serial sections 14 μm thick for rat and 10 μm thick for mouse tissue. The sections were thaw-mounted on gelatine-coated slides and air-dried at room temperature. The slides were stored at –20°C until use. Tissues were post-fixed for 2 min at room temperature in 4% formaldehyde (BDH Laboratory Supply, UK) and 0.03% picric acid in phosphate-buffered saline (PBS). Inactivation of endogenous peroxidase was carried out in 50% methanol and 0.3% H_2O_2 for 10 min. Blocking of nonspecific binding sites was achieved by preincubation with normal horse serum (NHS; Harlan Sera-Lab, UK) in PBS containing 0.05% thimerosal (Merthiolate; Sigma, Poole, 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. Antibodies to $P2X_{1-7}$, $P2Y_1$ and $P2Y_2$ receptors from rabbit were allowed to react with biotinylated donkey anti-rabbit IgG secondary antibody (Jackson Immunoresearch, Pa., USA) and detected with avidin-coupled horseradish-perioxidase/nickel-intensified 3,3′-diaminobenzidine (DAB) or with either Oregon green or avidin-coupled Texas red (Sigma). The P2X antibodies were obtained from Roche Bioscience (Palo Alto, Ca., USA). 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). The $P2Y_1$ antibody was kindly donated by Dr. C. Matute, Universidad del Pais Vasco, Spain, and obtained from Alomone (Alomone, Jerusalem, Israel). $P2Y_2$ antibody was from Alomone. Briefly, the sections were incubated overnight with the primary antibodies diluted to 5 μg/ml and 2.5 μg/ml (determined as optimal by previous titration) with 10% NHS in PBS containing 0.05% Merthiolate. For the $P2X_3$ antibody preparation, 0.2% of the detergent Triton X-100 was added. Subsequently the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Pa., USA) diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate for 30 min, followed by incubation with extravidin-horseradish peroxidase (Sigma) diluted 1:1,000 in PBS containing 0.05% Merthiolate for 30 min. All incubations were held at room temperature and separated by three 5-min washes in PBS. Finally, a freshly prepared colour reaction mixture containing 0.5% DAB, 0.1 M sodium phosphate, 0.004% NH_4Cl, 0.2% glucose, 0.04% nickel ammonium sulphate and 0.1% glucose oxidase was applied to the section for 5–10 min. The sections were washed, dehydrated, cleared in xylene and mounted using Eukitt (BDH, Poole, UK). Control experiments were performed using an excess of the appropriate homologue peptide antigen to absorb the primary antibodies and thus confirm a specific immunoreaction. In a series of experiments where a fluorescent marker was used, either Oregon green or Texas red (Sigma) both at a concentration of 1:100 were incubated for 1 h. These experiments followed a modified version of the protocol of Llewellyn-Smith et al. (1993; omitting the 0.02% of a saturated solution of picric acid, methanol-H_2O_2, and the nickel-DAB reaction steps). For anti-insulin, anti-glucagon and anti-somatostatin staining, a modified version of the protocol for P2X receptors was used. The primary antibody was guinea pig anti-insulin (Inestar Stillwater, Minn., USA) at a concentration of 1:1,000 and 1:2,000, and goat anti-glucagon and goat anti-somatostatin (Santa Cruz Biotechnology, Calif., USA), both at a concentration of 1:200. Instead of NHS, in anti-insulin experiments, normal goat serum (NGS) was used to block non-specific binding. A goat anti-guinea pig biotinylated secondary antibody (Sigma) was diluted in 1% NGS and incubated for 30 min, followed by incubation for 45 min in streptavidin-FITC. This experiment was also performed using Ni-DAB. The donkey anti-goat-FITC at a concentration of 1:100 was used as a secondary antibody for anti-glucagon and anti-somatostatin staining. The sections were viewed using an Edge R400 high-definition light microscope (Edge Scientific Instruments, Santa Monica, Calif., USA), Zeiss Axioplan, (Germany), and an Edge True-view 3D fluorescence microscope (Edge Scientific Instruments) with Kodak TMX 100 (ASA 100) black and white film, of Kodak PRD200X colour film.

Results

Expression of P2 receptors in islet cells during ageing

The expression of P2 receptors on rat islet pancreas during ageing varied substantially from no expression to high expression depending on the P2 receptor subtype studied. The neonate showed no visible signs of expressing $P2X_1$, (Fig. 1a) whereas islets from the mature 16-week-old rat had a dispersed pattern of expression, both within the β cell core and the surrounding mantle of cells (Fig. 1b). The cells expressing $P2X_1$ immunolabelling appeared to be in contact with neighbouring cells also expressing $P2X_1$ receptors. There was widespread $P2X_1$

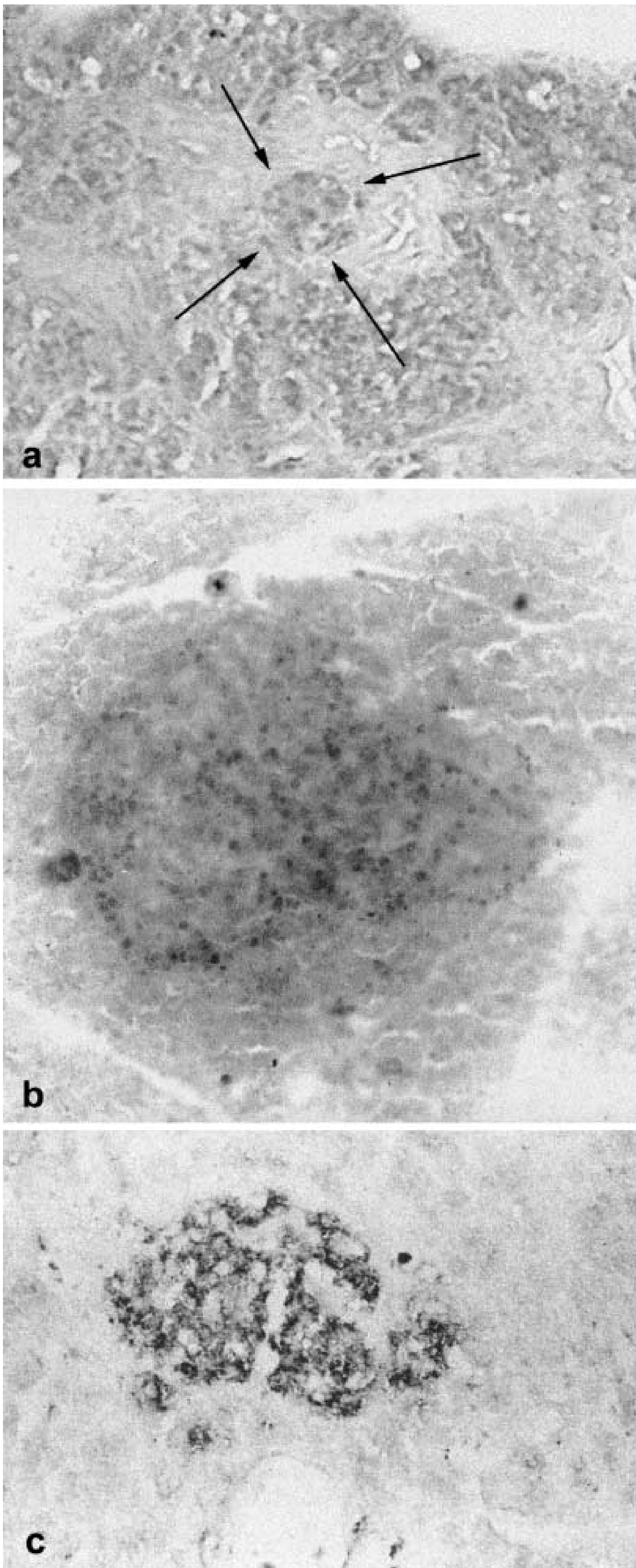

Fig. 1a–c $P2X_1$ staining in rat islet pancreas cells during development and ageing. **a** Three-day-old neonatal rat islet. Note the absence of $P2X_1$ receptor staining in the islet (*arrows*; Ni-DAB). **b** Mature 16-week-old rat islet. Note that there is some $P2X_1$ staining, where it shows uneven distribution in both the mantle and the core (Oregon green). **c** Aged 24-month-old rat pancreas. Note that there is strong and widespread immunoreactivity compared with the mature and neonatal islets. ×200

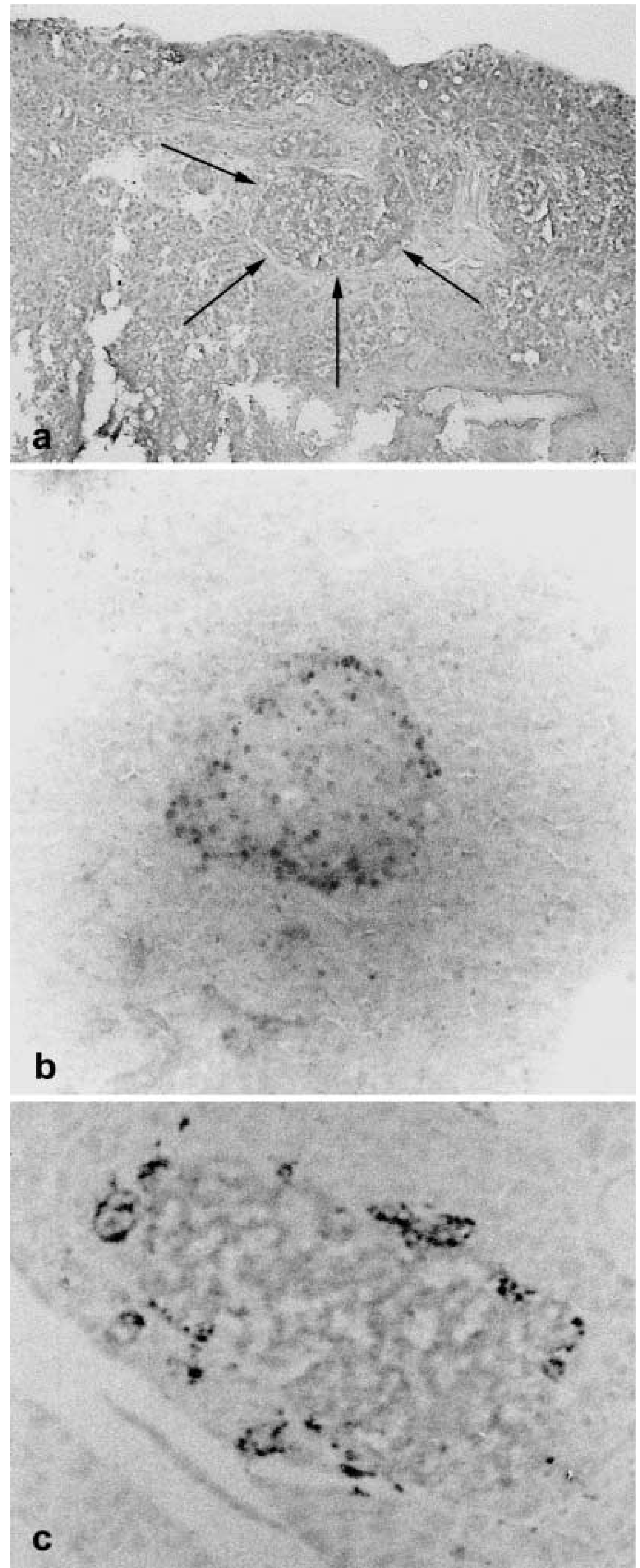

Fig. 2a–c $P2X_4$ staining in rat islet pancreas cells during development and ageing. **a** Three-day neonatal islet. Note that there is no staining in the islet (*arrows*; Ni-DAB). **b** The mature 16-week-old rat islet. Note that most of the $P2X_4$ receptor-staining has a peripheral distribution (Oregon green). **c** Aged 24-month-old rat islet. Note that there is a peripheral clustered distribution similar to the 16-week mature islet. ×200

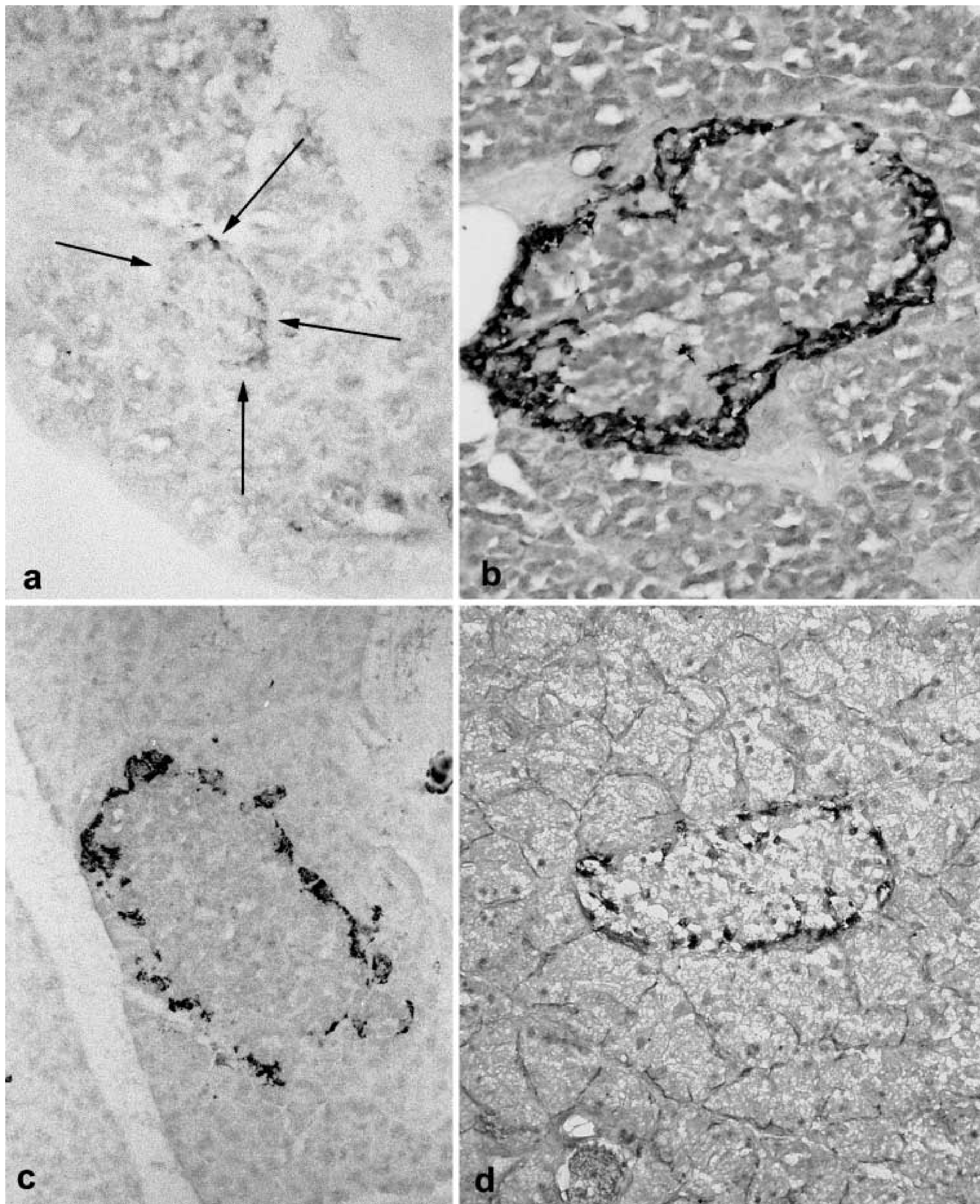

Fig. 3a–d $P2X_7$ staining in rat and mouse islet pancreas cells during development and ageing. **a** Three-day rat neonatal islet. Note that there is peripheral distribution of $P2X_7$ (*arrows*; Ni-DAB). **b** Mature 16-week-old rat islet. Note the peripheral distribution. **c** Aged 24-month-old rat islet. Note strong peripheral staining of $P2X_7$ receptors. **d** $P2X_7$ staining in 10-week-old mature Balb c mouse islet. As observed in rat tissue, the staining is peripheral. ×200

expression in the islets of the 24-month-old animal (Fig. 1c). Like $P2X_1$, $P2X_4$ receptors were not present or only present in very small amounts in the pancreatic islets of the neonate (Fig. 2a). The mature (16-week) pancreas had clusters of cells expressing $P2X_4$ scattered around its peripheral edge, forming an annulus (Fig. 2b), presumably consisting of A and F cells. The inner β cell mass did not appear to express $P2X_4$. There was expres-

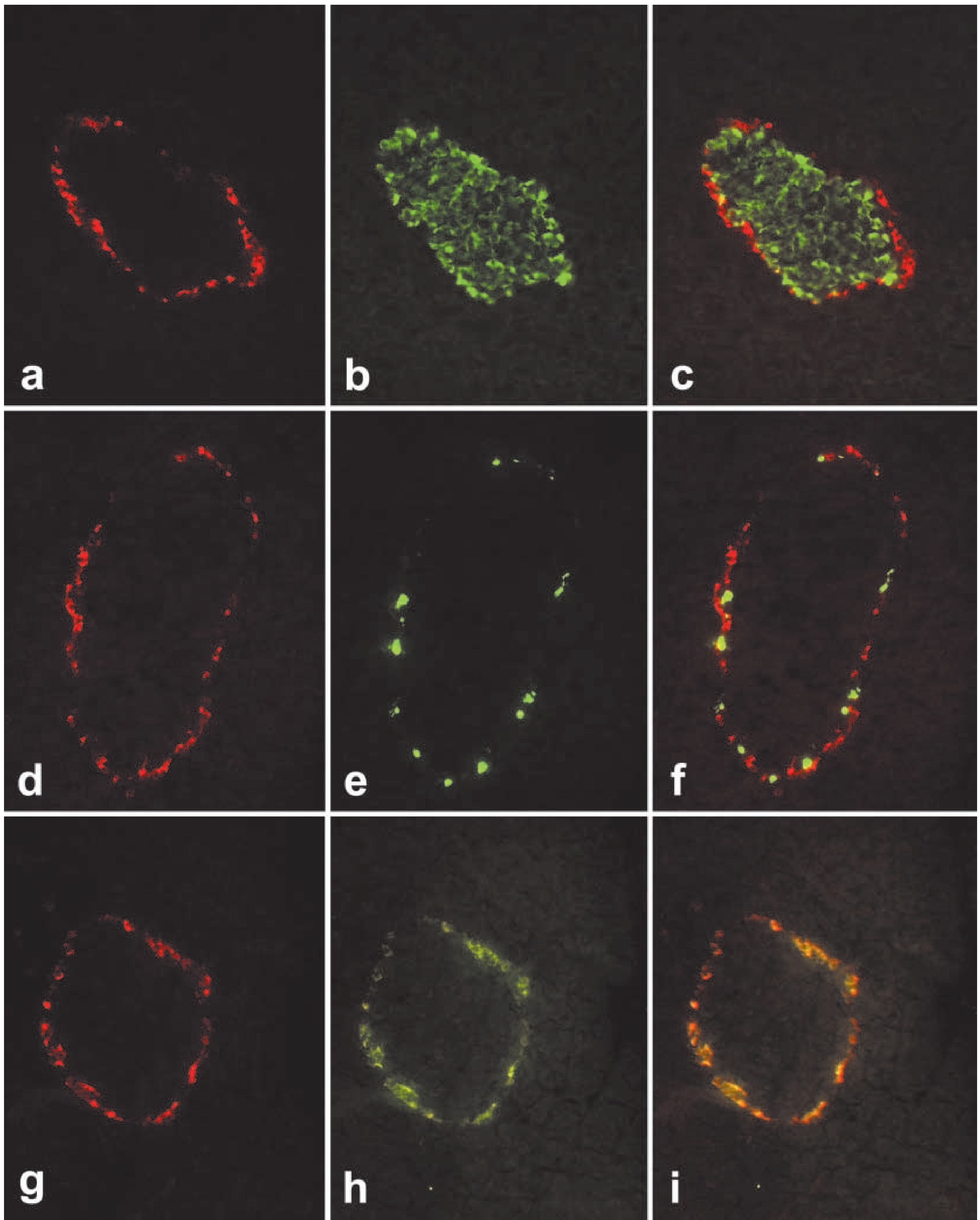

Fig. 4a–i Islet of Langerhans cells double-labelled for $P2X_7$ and insulin, somatostatin and glucagon. **a** $P2X_7$ immunostaining in islet cells from mature rat. **b** Insulin immunostaining showing β cells. **c** Superposition of **a** and **b** showing no colocalization between $P2X_7$ and insulin. **d** $P2X_7$ immunostaining. **e** Somatostatin immunostaining showing δ cells. **f** Superposition of **d** and **e**. Note that there are few cells double-labelled. **g** $P2X_7$ imunostaining. **h** Glucagon imunostaining showing α cells. **i** Superposition of **g** and **h**. Note the clear colocalization (*yellow*) between $P2X_7$ and glucagon. ×200

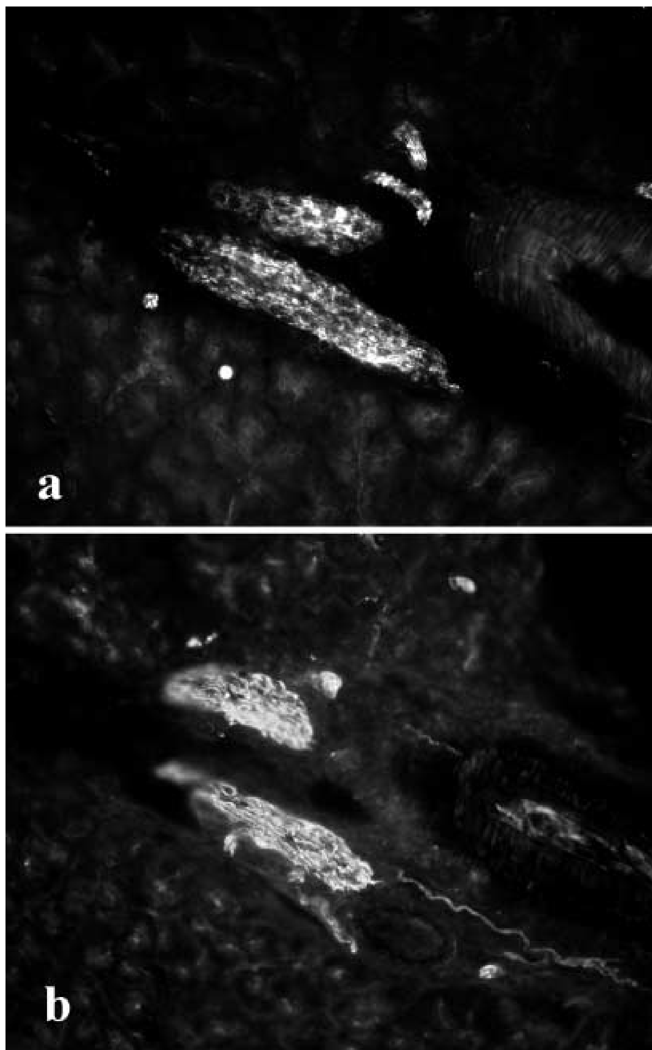

Fig. 5a, b P2X$_5$ receptors in pancreatic nerves. **a** P2X$_5$ receptor staining in the nerves of pancreas of 10-week-old mature Balb c mouse. **b** Nerves of an adjacent section stained with neurofilament antibody. ×400

sion of P2X$_4$ at the periphery of the 24-month-old islet (Fig. 2c), again in a clustered formation, and apart from one or two odd cells staining for P2X$_4$ there were no other populations that were P2X$_4$-positive. The expression of P2X$_7$ receptors showed the most consistent pattern of distribution in all of the age groups of rat and mouse studied. The outer periphery of the islet stained in an annular formation (Fig. 3). The amount and intensity of the staining did not appear to change during the process of ageing. We could not detect any staining for P2X$_2$, P2X$_3$, P2X$_5$ and P2X$_6$ in islet cells from rat or mouse. In order to determine which cells present in islets of Langerhans were positive for P2X$_7$, we performed experiments with double labelling for macrophages, α cells, β cells, δ cells and P2X$_7$ receptors. Only a few macrophages were found invading the islets (0–4 cells/islet; data not shown). This finding is in agreement with those of other workers, who have shown that the infiltration of macrophages in islet of normal pancreas is low (Fraser et al. 1997). The insulin-positive cells were a majority in the islet of Langerhans cell population (Fig. 4b) located in the core of the islets. The distribution of P2X$_7$ receptors has shown that there is no double labelling in β cells (Fig. 4b, c). The somatostatin-positive cells were found distributed mainly around the β cell core, but the double-labelling experiments showed that the δ cells, like β cells, did not express P2X$_7$ receptors. (Fig. 4d–f). The glucagon-positive cells were also found forming a ring around the islet. The α cells were more numerous than δ cells and, based on the double labelling with P2X$_7$, the α cells are islet cells that express P2X$_7$ receptors (Fig. 4g–i).

We observed a small degree of immunolabelling for P2Y$_2$ on the β cell core in all of the age groups of rat and mouse studied (data not shown). Staining for P2X$_5$ receptors on nerves in the mouse pancreas was established by staining of adjacent sections with neurofilament antibody (Fig. 5).

Expression of P2 receptors in duct cells

Immunostaining revealed widespread P2Y$_1$ and P2Y$_2$ expression on the membranes of a population of cells, which are presumably that of the duct system (Fig. 6). Blood vessels did not show P2Y$_1$ immunoreactivity (Fig. 6a) in the 3-day-old neonate. The greatest amount of P2Y$_1$ expression was found in the 3-day-old neonate (Fig. 6a), followed by the 16-week-old rat (Fig. 6b), while there was no P2Y$_1$ expression in the 24-month-old pancreas (Fig. 6c). P2Y$_1$ receptors were observed in both the large and the small ducts. In contrast, the expression of P2Y$_2$ receptors showed a consistent pattern of distribution in all of the age groups of rat tissue studied, being expressed in neonate, mature and aged rat duct cells of the pancreas (Fig. 6d–f). In neonate tissues, the P2Y$_2$ expression was observed in small and medium ducts, while, in mature and aged animals, the P2Y$_2$ receptors were expressed in small, medium and big ducts. P2Y$_1$ and P2Y$_2$ were also observed in mature mouse pancreatic duct cells.

Expression of P2 receptors in vascular tissue

Both P2X$_1$ and P2X$_2$ receptors were observed in vascular smooth muscle in both mature and aged rat vessels (Fig. 7a, b) and mature mouse vessels (Fig. 7e, f). This localization was confirmed by immunofluorescence for smooth muscle myofilaments (data not shown). The most obvious vessels were presumably arterial vessels with thick walls, which stained strongly for P2X$_2$. P2X$_1$ receptors were observed in neonate tissues, but P2X$_2$ was either absent or weakly expressed in those tissues (Fig. 7d). P2Y$_1$ and P2Y$_2$ receptors were observed in mature and aged rat blood vessel tissues (Fig. 8) but not in neonate rat. The expression of P2Y$_2$ receptors on

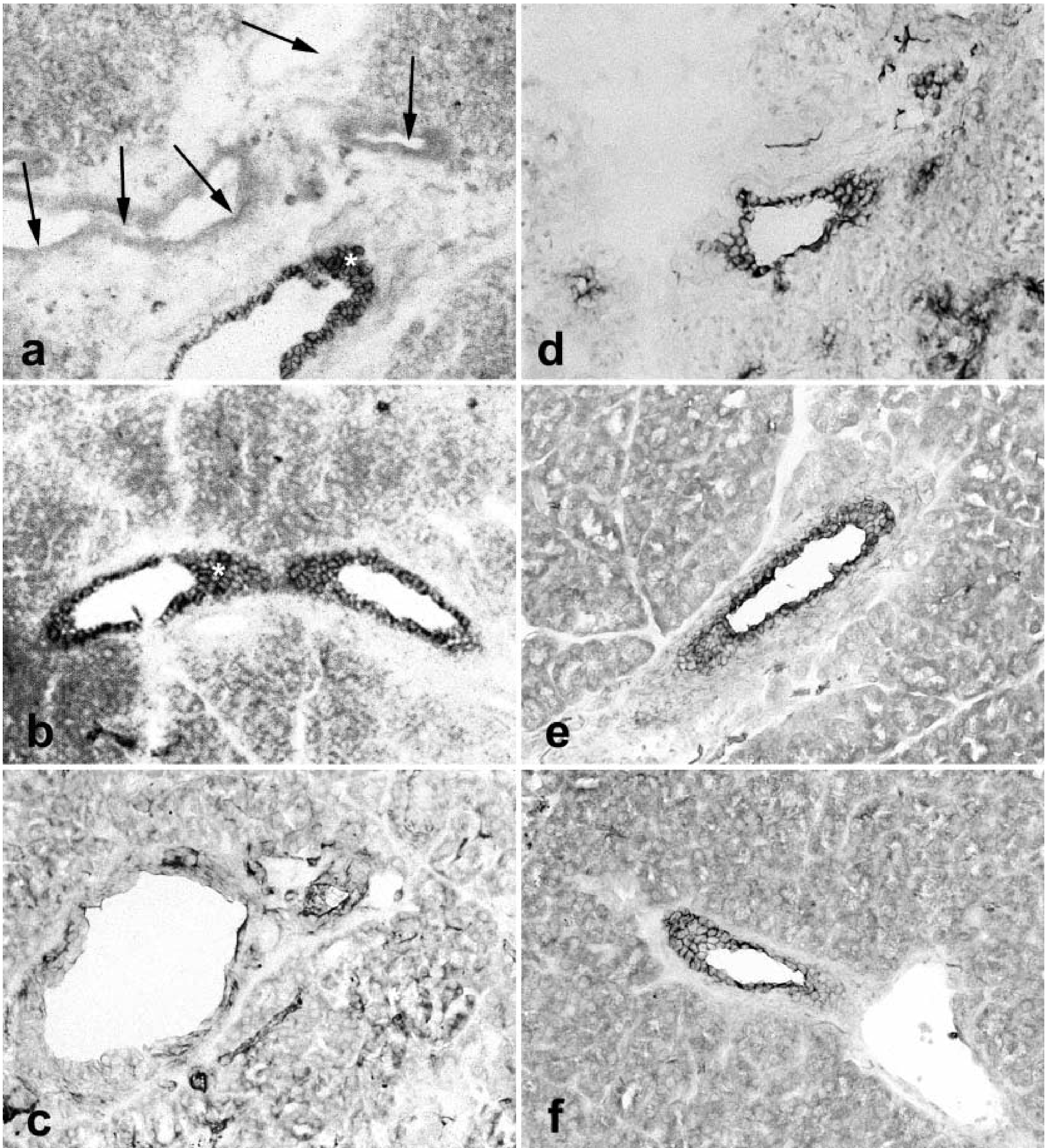

Fig. 6a–f $P2Y_1$ and $P2Y_2$ expression in pancreatic duct cells. **a** $P2Y_1$ immunofluorescent staining (Texas red) in the pancreatic duct of the 3-day-old neonate. Note that the staining is specific to the duct (*asterisk*), with the adjacent blood vessels (*arrows*) unstained. **b** $P2Y_1$ immunfluorescent staining (Texas red) in the duct cells of the 16-week mature rat. **c** The absence of $P2Y_1$ immunostaining in duct from aged 24-month-old rat. **d** $P2Y_2$ immunostaining in duct of 1-day-old neonate. **e, f** $P2Y_2$ immunostaining in pancreatic duct of mature and aged rat, respectively. ×200

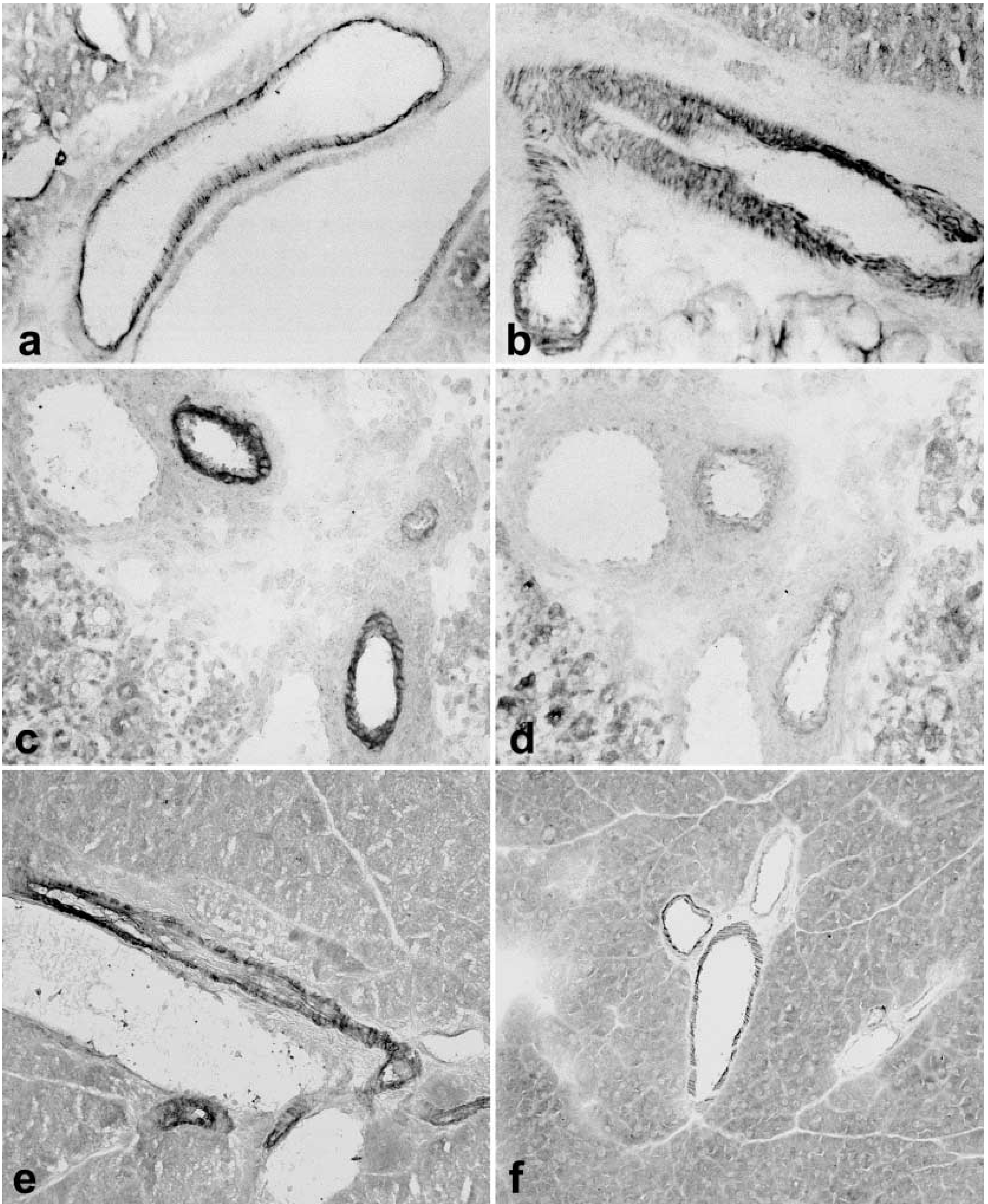

Fig. 7a–f P2X receptors in pancreas blood vessels. **a, b** $P2X_1$ and $P2X_2$ staining in the vascular smooth muscle of 16-week-old mature rat. **c, d** $P2X_1$ and $P2X_2$ staining in 1-day-neonate rat. Note there is little or no staining with $P2X_2$ in neonate tissues. **e**, **f** $P2X_1$ and $P2X_2$ staining in 10-week-old mature Balb c mouse. **a, b, e, f** ×400; **c**, **d** ×200

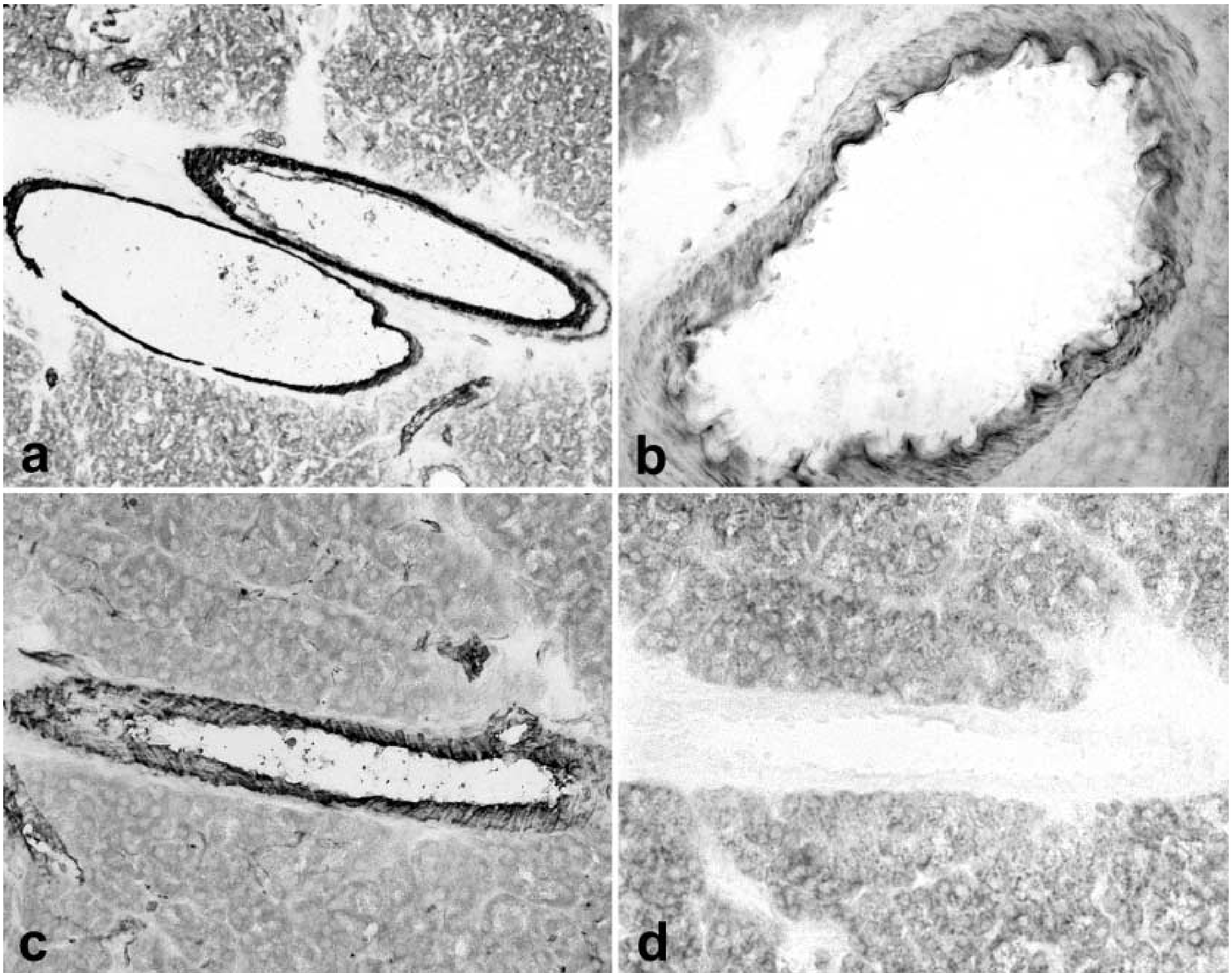

Fig. 8a–d P2Y receptors in pancreas blood vessels. **a**, **b** $P2Y_1$ and $P2Y_2$ staining in the vascular smooth muscle of 16-week-old mature rat, respectively. **c** $P2Y_1$ staining in the vascular smooth muscle of 24-month-old aged rat. **d** The immunoreactivity of a consecutive section shown in **c** is abolished after pre-absorption of $rP2Y_1$ antibody with $P2Y_1$ peptide. ×200

smooth muscle was restricted to a few big blood vessels. No other obvious P2X receptor expression was observed in vascular tissue, although the endothelium of the vessel wall produced strong autofluorescence. The results obtained from tissues from both mouse strains were similar.

Discussion

The expression of purinergic receptors in pancreas has been claimed by different groups mainly on the basis of functional studies. In 1963, Candela and Garcia-Fernandes were the first to report external ATP-stimulated insulin release in vitro from slices of rabbit pancreas. Several later reports described the effects of ATP in islet and duct cells (Hillaire-Buys et al. 1994; Hede et al. 1999; Luo et al. 1999). Our results have demonstrated immunohistochemically that $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ purinergic receptors exist in different regions of the pancreas. However, $P2X_3$ and $P2X_6$ were not present in any of the age ranges or conditions that were examined. Apart from regional differences, some P2 receptor subtypes in islet pancreas showed age-related changes that may suggest some functional roles. $P2X_1$ and $P2X_4$ expression increases from none, or very little, to widespread during the transition from neonate to old age. On the other hand, $P2Y_1$ expression changes in the opposite way, being expressed more in neonate pancreatic ducts, while being absent in the aged rat pancreas. We speculate that the expression of these P2 receptor subtypes is associated with cell turnover within the endocrine islets. In fact, upregulation of $P2X_1$ receptors has been associated with apoptosis in mouse thymocytes (Chvatchko et al. 1996), and $P2Y_1$ receptors have been associated with differentiation and cell turnover (Clifford et al. 1997; Park et al. 1997; Meyer et al. 1999). The expression of $P2X_7$ receptors was consistently located around islets during ageing. $P2X_7$ receptors have been implicated in apoptosis in different systems, including

macrophages (Coutinho-Silva et al. 2001), dendritic cells (Coutinho-Silva et al. 1999) and exfoliated epithelial cells (Gröschel-Stewart et al. 1999a, 1999b).The characterisation of $P2X_7$-positive cells in the pancreas has shown that the majority of islet cells expressing $P2X_7$ are α cells. To our knowledge this is the first report demonstrating $P2X_7$ receptor expression in α cells. In fact, to date only P1 adenosine receptors have been described in α cells, being associated with an increase in glucagon secretion (Weir et al. 1975; Loubatieres-Mariani and Chapal 1988). The identification of $P2X_7$ receptors on α cells may open a new avenue for understanding how extracellular ATP can modulate insulin secretion from β cells. Gap junctions have been identified between β cells and also between α and β cells (Orci 1982). Therefore, one signal initiated by activation of $P2X_7$ receptors on α cells may be propagated to neighbouring β cells to modulate their ATP response. Pancreas islets are richly innervated with both sympathetic and parasympathetic nerve terminals (Miller 1981) where ATP is a cotransmitter (Burnstock 1990) and an important constituent of both acetylcholine- and catecholamine-containing vesicles (Bertrand et al. 1986; Burnstock 1997). ATP may also be released by β cells (Detimary et al. 1996) and this release may be the signal for $P2X_7$ activation. Our results using double labelling for insulin and somatostatin and $P2X_7$ receptors show that most $P2X_7$-positive cells are neither insulin-positive nor somatostatin-positive. In addition, our findings do not support the hypothesis that the $P2X_7$-positive cells are macrophages that are found during normal islet functioning as scavengers of dying cells (Jansen et al. 1996).

The lack of $P2X_5$ receptors on duct cells from both rat and mouse tissues was an unexpected result, since the expression of $P2X_5$ receptors has been associated with proliferating and differentiating epithelial cells in skin (Gröschel-Stewart et al. 1999a), bladder and ureter (Lee et al. 2000) and gut (Gröschel-Stewart et al. 1999b). We expected to find the expression of $P2X_5$ on proliferating duct cells too. On the other hand, our results confirm the recent observation of an absence of $P2X_5$ receptors on duct cells measured by RT-PCR (Hede et al. 1999; Luo et al. 1999). This result indicates that the $P2X_5$ receptor is not involved in the proliferation or differentiation of pancreas duct cells. The expression of $P2Y_1$ and $P2Y_2$ observed in pancreas duct cells confirms the previous pharmacological studies that proposed their expression on related cells (Luo et al. 1999; Hede et al. 1999).

The expression of $P2X_1$ and $P2X_2$ receptors on vascular smooth muscle has been reported previously for coronary vessels, pulmonary, iliac, renal and femoral arteries (Nori et al. 1998), and now we have shown, for the first time, expression of both $P2X_1$ and $P2X_2$ receptors on pancreatic vascular smooth muscle. These two P2X receptors and the $P2Y_2$ receptor are the best candidates for mediating the vasoconstricting effects of extracellular nucleotides on pancreatic vascular tone (Chapal and Loubatières-Mariani 1983) and $P2Y_1$ on vasodilatation induced by adenosine-5′-*O*-2-thiodiphosphate (Hillaire-Buys et al. 1998) Under physiological conditions, sympathetic nerves may be the source of extracellular nucleotides which supply the blood vessels (de Gasparo et al. 1978; Hillaire-Buys et al. 1994).

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