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Molecular and Cellular Endocrinology 204 (2003) 141–154

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Molecular and Cellular Endocrinology

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P2X and P2Y purinoceptor expression in pancreas from streptozotocin-diabetic rats

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Received 16 August 2002; accepted 19 December 2002

Abstract

The expression of the nucleotide receptors $P2X_1$, $P2X_2$, $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_4$, in the pancreas of the streptozotocin-induced diabetic rat was investigated using immunohistochemistry. In diabetic animals, $P2X_7$ receptor expression, normally located in the outer periphery of the islet, was increased and located inside the islet. Double-labelling experiments, using antibodies raised against insulin, somatostatin and glucagon, showed, for the first time, an increase in immunostaining for $P2X_7$ receptors on islet glucagon-containing α cells (which had migrated to the interior), while no $P2X_7$ receptors were found in β and δ cells. $P2Y_1$ receptors were present in intra-islet capillaries, while $P2Y_4$ receptors were found on both α and β cells. $P2Y_1$ and $P2Y_2$ receptor expression was also found in pancreatic duct cells and $P2X_1$, $P2X_2$, $P2Y_1$ and $P2Y_2$ receptors were identified in small blood vessels.

Keywords: Diabetes; Purinoceptors; Pancreas; Adenosine triphosphate; Streptozotocin; Immunocytochemistry

1. Introduction

The glucose analog streptozotocin (STZ), is widely used experimentally to induce diabetes mellitus (Wilson and Leiter, 1990). A single high dose injection of STZ can induce diabetes in rats and direct cytotoxic effects on islet β cells have been reported in both rat and mouse within 24–72 h of administration (Bolaffi et al., 1986; Schnedl et al., 1994; Strandell et al., 1988; Like and Rossini, 1981). In addition, during STZ-induced diabetes, various nerves, organs and tissues may be affected; vascular dysfunctions have been described in the eye, heart and kidney (Vinik et al., 2000) with altered responses to various vasodilator or vasoconstrictor agents during the diabetic state (Kamata et al., 1989; Mulhern and Docherty, 1989).

Exogenous adenosine tri- and di-phosphate nucleotides modulate the functions of the pancreas (Candela and Garcia-Fernandes, 1963; Levine et al., 1970; Loubatières-Mariani et al., 1979; Chapal and Loubatières-Mariani, 1981). It has been demonstrated that exogenous adenosine and its nucleotides modulate insulin secretion. While adenosine triphosphate (ATP) stimulates insulin secretion, adenosine inhibits insulin secretion from β cells and stimulates glucagon secretion from α cells (Loubatières-Mariani and Chapal, 1988). Extracellular ATP has been shown to stimulate insulin secretion by raising $[Ca^{2+}]_i$ in insulin-secreting cell lines (Geschwind et al., 1989; Kindmark et al., 1991), in isolated rat and human islets (Kindmark et al., 1991; Fernandez-Alvarez et al., 2001) and in perfused rat pancreas (Bertrand et al., 1991). Also, other selective P2 purinoceptor agonists have been shown to increase insulin secretion and decrease glycemia in vivo (Ribes et al., 1988; Hillaire-Buys et al., 1993). In addition, nucleotides can regulate vascular tone, probably following release from sympathetic nerves (Chapal and Loubatières-Mariani, 1983; Hillaire-Buys et al., 1998).

P2 receptors mediate nucleotide function to regulate many cellular processes. These receptors are 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. To date, seven members of the P2X

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0303-7207/03/$ - see front matter
doi:10.1016/S0303-7207(03)00003-0

($P2X_{1-7}$) family and eight subtypes of mammalian P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) have been cloned (Jacobson et al., 2000; Hollopeter et al., 2001; Communi, et al., 2001; Abbracchio et al., 2003). The presence of P2X and P2Y receptors in pancreatic tissues has been determined largely using a pharmacological approach (Luo et al., 1999; Hede et al., 1999; Stam et al., 1996). Based on the measurement of $[Ca^{2+}]$ in microperfused intralobular ducts and RT-PCR analysis, it has been proposed that $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors were present on pancreatic duct cells (Luo et al., 1999; Hede et al., 1999).

Exogenous ATP may elicit both vasoconstriction (via activation of a P2X receptor on vascular smooth muscle), and vasodilatation (via P2Y receptors on the endothelium) of pancreatic blood vessels. Extracellular nucleotides may also exert effects on pancreatic duct cells (Chan et al., 1996; Christoffersen et al., 1998; Luo et al., 1999). Some of the effects described above are preserved in diabetic rats (Hillaire-Buys et al., 1992; Tang et al., 1996).

$P2X_7$ has been shown to mediate apoptosis in various cell types (Morelli et al., 2001; Coutinho-Silva et al., 1999; Gröschel-Stewart et al., 1999a). Since it has been demonstrated that the damage of the pancreatic islet observed in diabetes may involve apoptosis (Bolaffi et al., 1986; Saini et al., 1996), it was of interest to examine whether $P2X_7$ might be associated with cell destruction within the islet. In the present study, we investigated, using immunohistochemistry, the expression of $P2X_7$ in the diabetic islet over short- and long-term periods. The localization of $P2X_1$, $P2X_2$, $P2Y_1$, $P2Y_2$ receptors, previously observed in normal rat pancreas (Coutinho-Silva et al., 2001a) and $P2Y_4$ were also examined in the pancreas of STZ-induced diabetic animals.

2. Materials and methods

2.1. Animals

Breeding, maintenance and sacrificing 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. This study was carried out with adult male Wistar rats weighing between 400 and 450 g. Diabetes was induced in a group of 24 rats by a single i.p. injection of STZ (Sigma, St. Louis, MO) dissolved in 20 mM citrate buffer at pH 4.6. The dose administered was 65 mg/kg body weight. The same number of control animals received the vehicle alone. Animals were kept at a constant 12 h/12 h light–dark cycle with free access to food and water. Animals were maintained for 1 day, 3 days, 2 weeks, 8 weeks and 12 weeks and were then sacrificed by exposure to an increasing dose of carbon dioxide. Death was confirmed by exsanguination and the pancreas removed. Eleven treated rats were sacrificed at day 1, 3 at day 3, 8 at 2 weeks and 4 rats were sacrificed after 8 and 12 weeks of STZ injection. Diabetes normally took more than 3 days to develop. Blood samples were taken at sacrifice for plasma–glucose analysis using an automated glucose monitor at stages from 2 to 12 weeks. Only rats with glucose levels greater than 28 mM were used for analysis of diabetic tissue.

2.2. Immunohistochemistry

2.2.1. Tissue handling

The pancreas from each rat was removed, placed in Hanks' balanced salt solution (HBSS), embedded in OCT tissue compound (BDH), progressively frozen in *iso*-pentane (pre-cooled in liquid nitrogen) and then stored in liquid nitrogen. Cryostat sections were cut as sets of serial sections of 12-μm thickness. The sections were thaw-mounted on gelatine-coated slides, air-dried at room temperature and 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). Endogenous peroxidase was inactivated by incubation for 10 min with 0.3% H_2O_2 prepared in 50% methanol. Blocking of non-specific binding sites was achieved by preincubation with normal horse serum (NHS; Harlan Sera-Lab., UK) in PBS containing 0.05% Merthiolate (Sigma) at room temperature for 20 min, as described in detail by Llewellyn-Smith et al. (1993).

2.2.2. Immunostaining

An indirect immunohistochemical and immunofluorescent method with two layers of antibodies was used. Antibodies for $P2X_1$, $P2X_2$ and $P2X_7$ 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). 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 $P2Y_1$, $P2Y_2$ and $P2Y_4$ antibodies were obtained from Alomone (Alomone Lab. Ltd, Jerusalem, Israel). Briefly, the sections were incubated overnight with primary antibodies diluted to 5 and 2.5 μg/ml (determined as optimal by previous titration) with 10% NHS in PBS containing 0.05% Merthiolate. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch)

diluted 1:500 in 1% NHS in PBS containing 0.05% Merthiolate for 30 min, followed by incubation with ExtrAvidin-horseradish peroxidase (Sigma) diluted 1:1000 in PBS containing 0.05% Merthiolate for 30 min. All incubations were carried out 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 sections for 5–10 min. Sections were then 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, sections were incubated for 1 h with either streptavidin-conjugated Oregon green or Texas Red (Sigma) both at a concentration of 1:100. These experiments were performed using a modified version of the protocol of Llewellyn-Smith et al. (1993) (omitting 0.02% of a saturated solution of picric acid, inactivation of endogenous peroxidase, and the Ni–DAB reaction steps). For $P2X_7$ receptor and Mac-1 double-labelling, the samples were simultaneously incubated overnight with

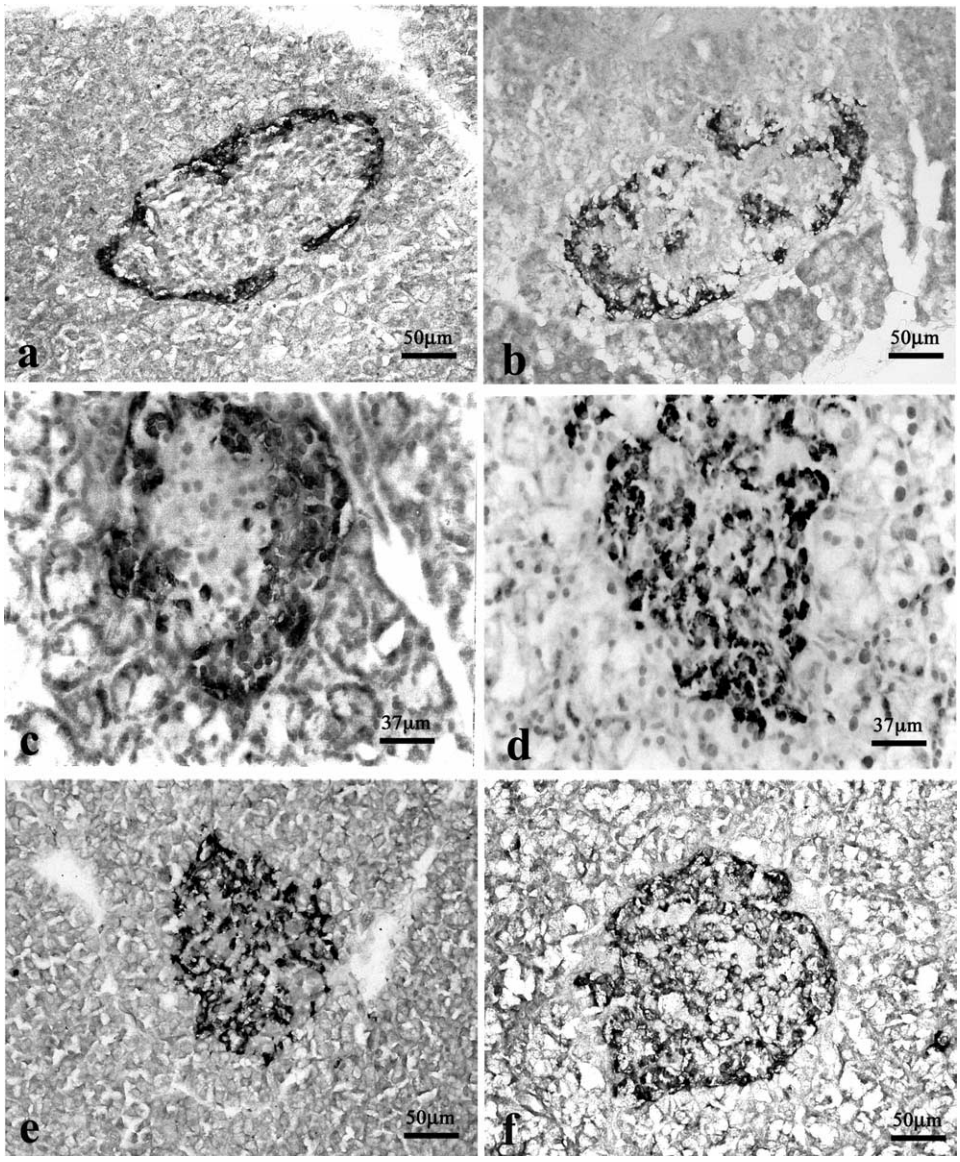
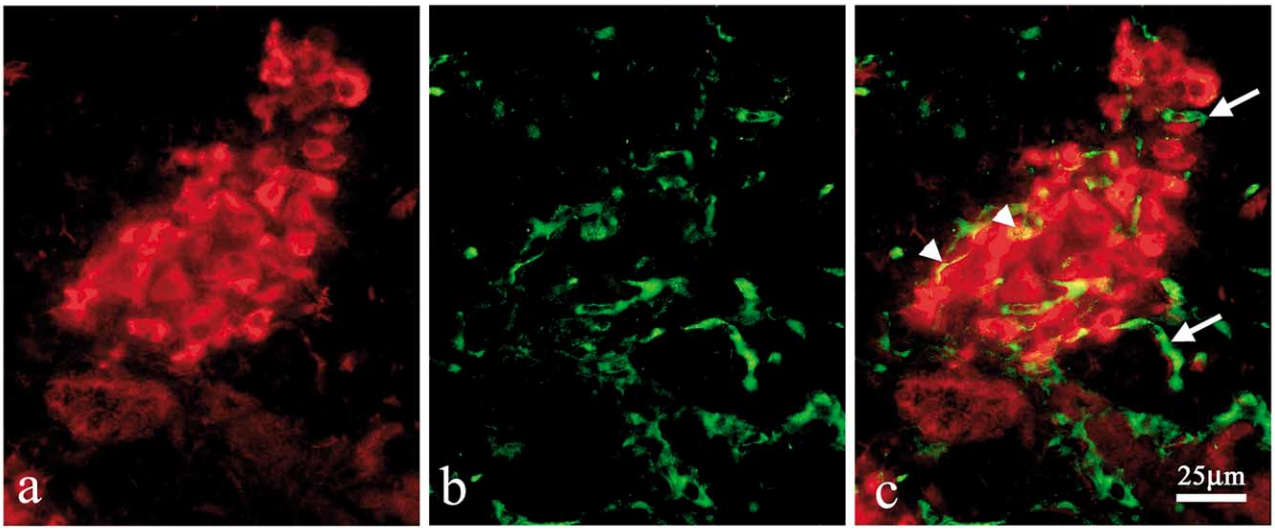
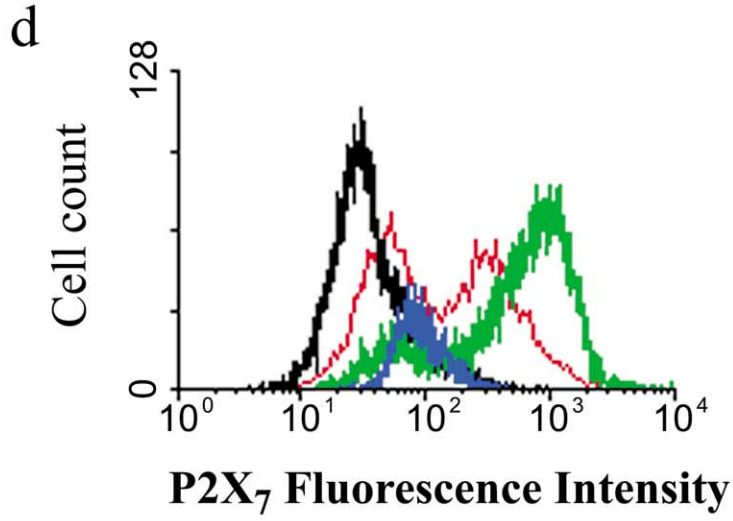


Fig. 1. STZ treatment induced changes in $P2X_7$ receptor expression in the islet of Langerhans. (a) $P2X_7$ receptor staining in normal rat islet pancreas cells. Note the positive immunostaining in cells at the periphery of the islet. (b) Twenty-four hours after STZ treatment. Note that, in addition to distribution around the periphery, there is some immunostaining in the islet core. (c) Seventy-two hours diabetic rat islet. Note immunostaining in the islet core is increased. (d) Two-week diabetic rat islet. (e) 8-week diabetic rat islet. (f) Twelve-week diabetic rat islet. Note clear, widespread immunostaining in (d) and the strong and widespread immunostaining on the islet located in both the mantle and the core in (e) and (f).


a
b
c
25µm


d
Cell count
128
0
10^0
10^1
10^2
10^3
10^4
P2X7 Fluorescence Intensity

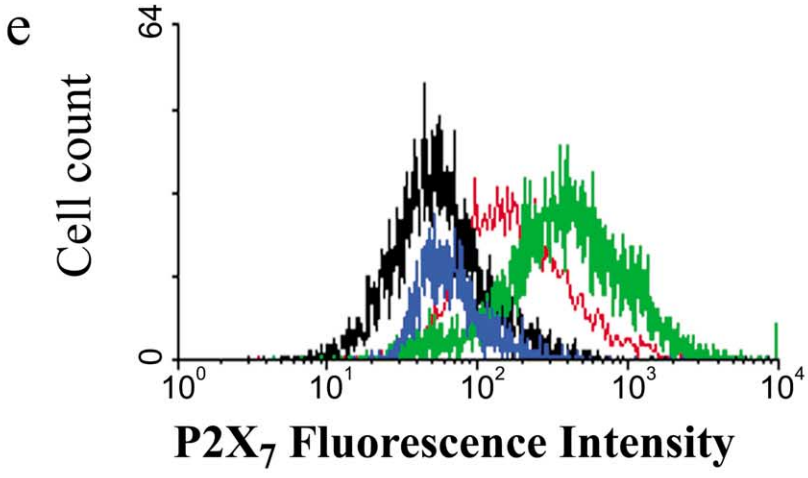

e
Cell count
64
0
10^0
10^1
10^2
10^3
10^4
P2X7 Fluorescence Intensity

Fig. 2

anti-$P2X_7$ antibody (5 μg/ml), and mouse anti-rat CD11b/Mac-1 antibody (10 μg/ml; Serotec, UK). The samples were then incubated with goat anti-mouse IgG FITC conjugate (1:100; Serotec), followed by $P2X_7$ receptor immunostaining (as described above) using Texas Red.

For anti-insulin, anti-glucagon and anti-somatostatin staining, a modified version of the protocol for P2X receptors was used. The primary antibodies used were guinea-pig anti-insulin (Inestar Stillwater, Minn.) at a concentration of 1:1000 and 1:2000, and goat anti-glucagon and goat anti-somatostatin (Santa Cruz Biotechnology, CA) both at a concentration of 1:200. In anti-insulin experiments, normal goat serum (NGS) was used to block non-specific binding, instead of NHS. A goat anti-guinea-pig biotinylated secondary antibody (Sigma) was diluted in 1% NGS and applied to slides for 30 min, followed by incubation with streptavidin–FITC for 45 min. This experiment was also performed using Ni–DAB. Donkey anti-goat-FITC, at concentration of 1:100, was used as a secondary antibody for anti-glucagon and anti-somatostatin staining. The microscopes used were a Zeiss Axioplan, (Zeiss, Germany), and an Edge True-View 3D fluorescence microscope (Edge Scientific Instruments, Santa Monica, CA). Photographs were taken with Kodak TMX 100 (ASA 100) black and white film, or Kodak PRD200X colour film.

2.2.3. Preparation of macrophages from spleen and peritoneum

Macrophages were obtained by lavage of the i.p. cavity with cold BSS medium from 24 h-treated STZ rats. Cells were transferred to HBSS medium containing 5% foetal bovine serum for immediate immunostaining assays. Spleens were collected from control and STZ-treated rats, 24 h after STZ injection. The splenocytes were then gently removed by mechanical dissociation and re-suspended in HBSS. The erythrocytes were removed and the mononuclear cells enriched by centrifugation on a Ficoll density gradient Histopaque 1083 (Sigma). Cell viability was over 95% in all cases.

2.2.4. Flow cytometry assays

Peritoneal and splenocyte cell numbers were adjusted to 10^6 cells/eppendorf, washed twice with HBSS and incubated with BSS containing 5% NGS, on ice, for 20 min. Cells were then either incubated with FITC-conjugated mouse anti-rat CD3 (1:100; Serotec), FITC-conjugated mouse anti-rat B220 (1:100; Serotec) or FITC-conjugated mouse anti-rat Mac-1 (10 μl neat antibody/10^6 cells; Serotec) for 30 min. The samples were then washed and fixed in fresh 4% paraformaldeyde (Sigma) for 10 min on ice and then extensively washed in cold HBSS. The rabbit anti-rat $P2X_7$ antibody (Roche Bioscience) was diluted in HBSS 0.1% saponin medium and applied to the samples at a concentration of 0.1 μg/10^6 cells, overnight at 4 °C. The samples were then washed 3 times in HBSS and incubated with phycoerythrin (PE)-goat anti-rabbit monoclonal antibody (Caltag Lab., Burlingame, CA) at a concentration of 1 μg/10^6 cells for 30 min. PE labels with fluorescence at a different wavelength to FITC. The cells were then washed 3 times in HBSS, re-suspended in PBS and samples of 10^4 cells/animal were analysed on a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA). Negative control experiments were performed by preabsorbing the $P2X_7$ antibodies with its appropriate homologue peptide antigen and/or by omission of the primary $P2X_7$ antibodies. Cells were initially gated by forward and side scatter and then by cell type-specific antibodies for macrophages (Mac-1), T lymphocytes ($CD3^+$). Data were quantified using the program WinMDI (version 2.7) which calculates mean fluorescence intensity (MFI), an index of number of receptors/cell.

2.2.5. Statistical analysis

Statistical analysis was performed using unpaired Students' t-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Expression of $P2X_7$ receptors in islets of Langerhans cells from diabetic rats

The islets of Langerhans from diabetic animals were fewer than those of normal animals and appeared atrophied. $P2X_7$ receptor expression in diabetic islets

Fig. 2. $P2X_7$ receptor protein expression in macrophages during STZ treatment. (a–c) Double-labelling with $P2X_7$ receptor and a macrophage marker, Mac-1 on the islet from 8-week diabetic rat. (a) $P2X_7$ receptor immunostaining (red). (b) Macrophage immunostaining (green). Note the presence of macrophages around and inside the islet. (c) Co-localization of $P2X_7$ and the macrophage marker. Note that most of the macrophages are weakly stained or negative for $P2X_7$ (arrows) but a few co-express $P2X_7$ and Mac-1 (arrowheads). Scale bar applies to (a–c). (d and e) Fluorescence histograms of $P2X_7$ receptor expression of (d) peritoneal and (e) spleen macrophages 24 h after STZ treatment. Cells were initially gated by forward and side scatter and then by cell type-specific antibodies for macrophages (Mac-1), T lymphocytes ($CD3^+$) (d) is a representative trace of peritoneal macrophages from one control rat (red) and one STZ-treated rat (green). For both (d) and (e) the negative control is indicated by the black trace (STZ-treated) and blue trace (normal rat). The experiments were repeated 6 times (e) is a representative trace of spleen macrophages from one control rat (red) and one STZ-treated rat (green). The experiments were repeated 6 times. Note that there is a clear difference in $P2X_7$ fluorescence intensity between normal and treated rats (i.e. the superimposed peaks are different).

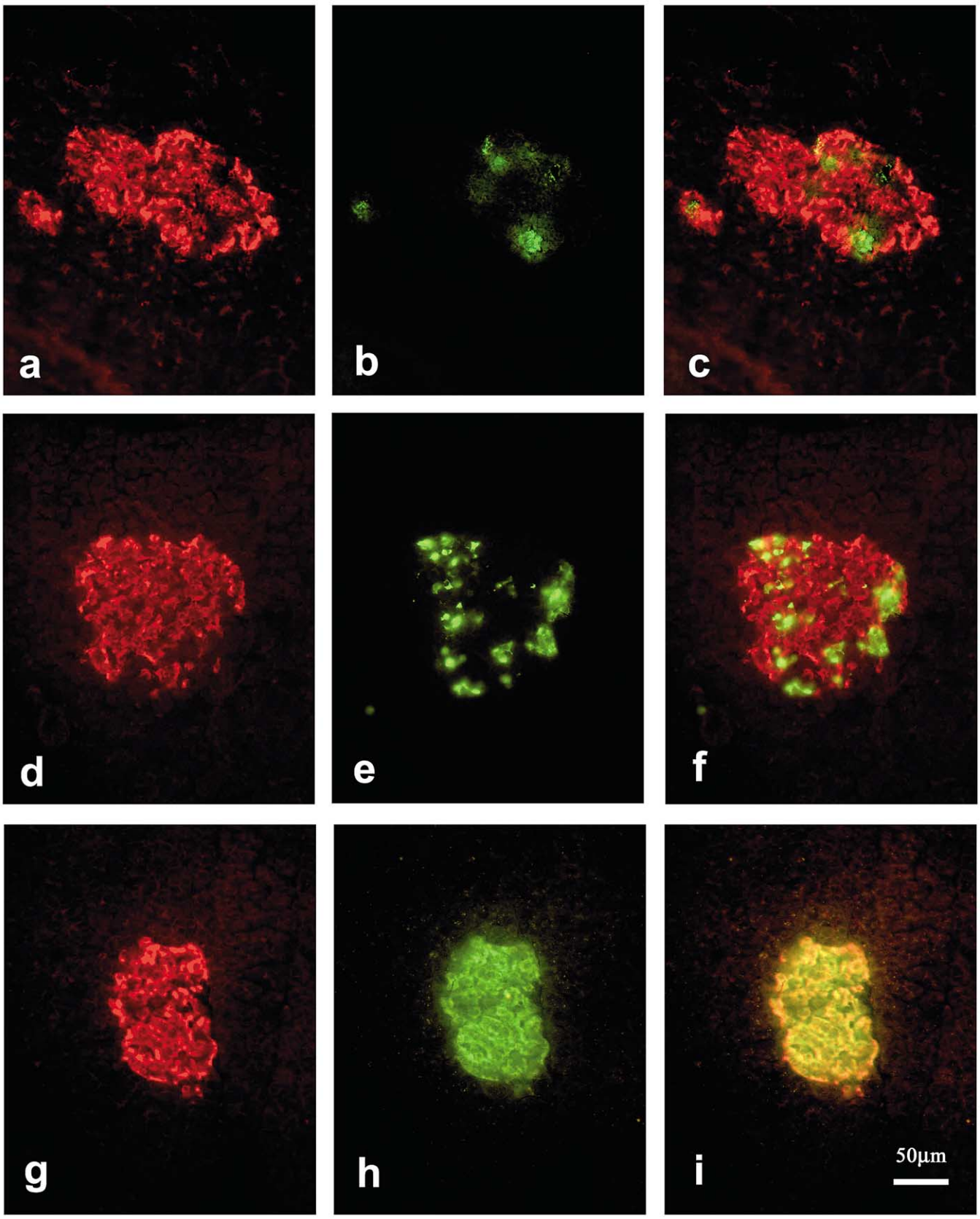


Fig. 3

was completely different from normal rat islets. In normal rat Langerhans islets, $P2X_7$ receptor expression was consistently seen only in the outer periphery of the islets, (Fig. 1a). In the majority of sections, these rims had a typical uniform thickness of 1–2 cells, although in a few sections, parts of these islet rims were as much as 3–4 cells thick. In contrast, the islets of Langerhans from diabetic animals had a much more scattered distribution of $P2X_7$ receptors. After 72 h, some $P2X_7$-positive cells were located within the core of islets (Fig. 1c). After 2 weeks (Fig. 1d), the number of positively-stained cells in the core was increased. At 8 weeks (Fig. 1e) and 12 weeks (Fig. 1f) after STZ injection, positive cells were spread throughout all remaining islets.

Diabetes normally takes more than 3 days to develop (Bolaffi et al., 1986), however we collected tissues after 24 h of STZ injection to investigate whether changes in expression of $P2X_7$ receptors on islets are already apparent at this time. The results show that as early as 24 h after STZ injection, $P2X_7$-positive cells are observed at the core of the islet of Langerhans. This disruption of the normal pattern of $P2X_7$ staining was observed in tissues taken from 4 of 5 animals (Fig. 1b).

3.2. Expression of $P2X_7$ receptors in macrophages

In normal animals, macrophages were easily detected in the exocrine tissue of the pancreas and also in small quantities (1–3 cells) either surrounding or infiltrating the pancreatic islets. The number of macrophages was increased around and inside the islets in 8 weeks diabetic animals (5–18 cells) but double-labelling immunostaining with $P2X_7$ receptors showed that only a few $P2X_7$-positive cells in the islets were macrophages (Fig. 2a). In fact the Mac-1-positive, $P2X_7$-positive macrophages present showed weaker staining than the majority of non-macrophage, Mac-1-negative, $P2X_7$-positive cells inside the islet. Finally, we compared the expression of $P2X_7$ receptor protein in peritoneal and splenic macrophages from animals 24 h after STZ-treatment, with untreated animals in flow cytometry analysis. Fig. 2d–e illustrates the flow cytometry histograms of $P2X_7$ receptor expression in macrophages from the peritoneum and spleen of STZ-treated vs. untreated rats. The MFI (an index of receptor number per cell) of $P2X_7$ receptor expression on macrophages was 524 ± 41 ($n = 6$ animals) in peritoneum macrophages from STZ-treated rats vs. 363 ± 39 ($n = 4$ animals; $P = 0.02$) from untreated animals and 412 ± 31 ($n = 6$ animals) in spleen macrophages from treated rats vs. 281 ± 12 ($n = 4$ animals; $P = 0.01$) from untreated animals. Therefore, flow cytometry analysis showed that there was a significant increase in $P2X_7$ expression on macrophages from peritoneum or spleen 24 h post-STZ injection (Fig. 2d–e).

3.3. Expression of $P2X_7$ receptors in different islet cell types

To ascertain which endocrine cell type was positive for $P2X_7$ in the islet pancreas, we performed double-labelling immunostaining for $P2X_7$ and insulin (a marker for β cells), $P2X_7$ and somatostatin (a marker for δ cells) and $P2X_7$ and glucagon (a marker for α cells) in 8-week diabetic rats. We identified a few insulin-producing cells in the diabetic tissues that were not stained for $P2X_7$ markers (Fig. 3a–c). Somatostatin-positive cells were spread throughout the islet but were not co-localized with $P2X_7$-positive cells (Fig. 3d–f). In contrast, we observed a massive increase in glucagon-positive cells on diabetic islet of Langerhans and this co-localized with $P2X_7$-positive cells (Fig. 3g–i).

3.4. Expression of $P2Y_1$ and $P2Y_4$ on islet of Langerhans

$P2Y_1$ and $P2Y_4$ receptor expression was observed in the islet of Langerhans of both normal and diabetic animals at all stages. $P2Y_1$ staining was sparsely distributed in the islet (Fig. 4a). Double-labelling with capillary markers showed that the endothelial cell intra islets are $P2Y_1$-positive cells in this site. In contrast, $P2Y_4$ expression was widespread both in the β cell core and the surrounding mantle of cells in islets from normal rats (Fig. 4b). The expression of $P2Y_4$ was also widespread in the majority of islets from diabetic animals. However, in some experiments we observed a slight increase in $P2Y_4$ expression on islet cells from long-term diabetic animals (Fig. 4c).

3.5. Identity of islet cells expressing $P2Y_4$ receptors

We performed double-labelling immunostaining for $P2Y_4$ and insulin, $P2Y_4$ and somatostatin and $P2Y_4$ and glucagon in both normal and diabetic rat pancreas. Our data demonstrated that the majority of β cells (Fig. 5a–c) and some α cells express $P2Y_4$ receptors in normal

Fig. 3. Islet of Langerhans double-labelling with $P2X_7$ receptor (a, d and g) and insulin (b and c), somatostatin (e and f) and glucagon (h and i) in 8 week diabetic animals. (a) $P2X_7$ receptor immunostaining in islet cells. (b) Insulin immunostaining showing β cells. (c) Indicates (a) and (b) co-localization showing no double-labelling between $P2X_7$ receptor and insulin. (d) $P2X_7$ receptor immunostaining. (e) Somatostatin immunostaining showing δ cells. (f) Shows (d) and (e) co-localized. Note that there are no double-labelled cells. (g) $P2X_7$ receptor immunostaining. (h) Glucagon immunostaining showing α cells. (i) Shows (g) and (h) double-labelled. Note the widespread immunostaining for glucagon-positive cells and clear co-localization (yellow) of the $P2X_7$ receptor and glucagon. Scale bar applies to all panels.

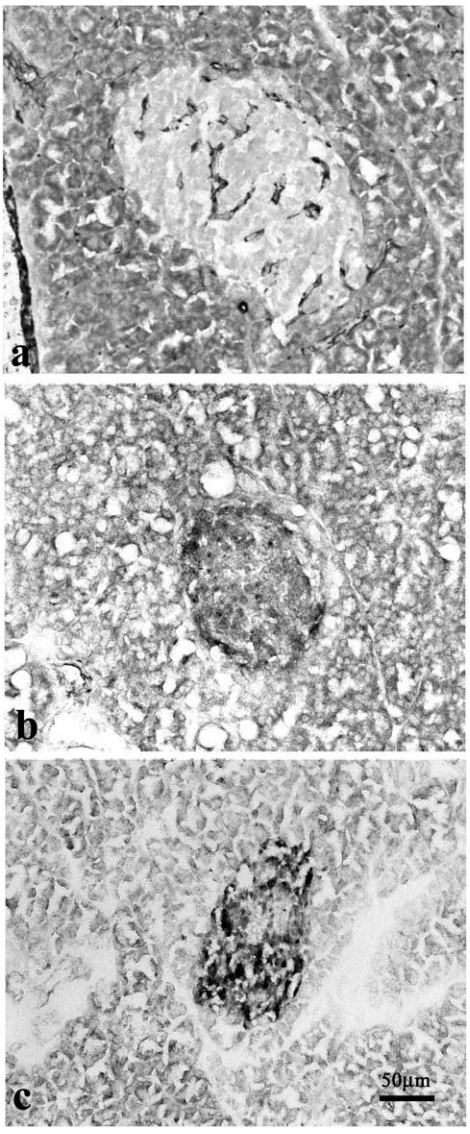


Fig. 4. $P2Y_1$ receptor (a) and $P2Y_4$ receptor staining in normal rat islet pancreas cells (b) and STZ-induced diabetic rat (c). (a) $P2Y_1$ receptors in normal rat islet. Note the widespread immunostaining in capillaries. (b) $P2Y_4$ receptor in normal rat islet. Note that $P2Y_4$ receptor immunostaining is widespread at the core and surrounding mantle of cells. (c) $P2Y_4$ receptor expression in 8-week diabetic rat islet. Note the similar distribution of immunostaining seen in (c) but with some cells more intensely stained. Scale bar applies to all panels.

pancreas. In long-term diabetic animals, $P2Y_4$ receptors were found to be expressed in the few remaining β cells (Fig. 5d–f), some α cells (Fig. 5j–l) and some infiltrating cells, possibly macrophages, (Fig. 5l; red cells). The δ cells are not immunolabelled for $P2Y_4$ either in normal or diabetic animals (Fig. 5g–i).

3.6. Expression of $P2Y_1$ and $P2Y_2$ in duct cells

Positive immunostaining for $P2Y_1$ and $P2Y_2$ receptors was observed in pancreas duct cells from normal adult rat (Fig. 6). Immunostaining for $P2Y_2$ receptor expression was stronger and more widespread than $P2Y_1$ and was observed in the majority of ducts (Fig. 6a and b). Diabetes (short- and long-term) did not affect the pattern of expression of either receptor (Fig. 6c and d).

3.7. Immunolocalization of P2X and P2Y receptors in blood vessels from normal and diabetic rat pancreas

During STZ-induced diabetes there is muscular atrophy and altered nervous system response. We therefore investigated the expression of P2X and P2Y receptors in smooth muscle from pancreatic blood vessels. In normal rat tissue, we observed positive immunostaining for $P2X_1$, $P2X_2$, $P2Y_1$ and $P2Y_2$ in blood vessels (Fig. 7a, c and e). The staining observed was present mainly in smooth muscle with $P2X_1$, $P2X_2$ and $P2Y_1$ observed in the majority of blood vessels and $P2Y_2$ receptor expression detected mainly in larger blood vessels. A similar pattern of staining for $P2X_1$, $P2X_2$, $P2Y_1$ and $P2Y_2$ was observed in blood vessels taken from long-term diabetic animals (8 and 12 weeks after STZ treatment; Fig. 7b, d and f). All data is summarised in Table 1.

4. Discussion

Nucleotides have been shown to promote insulin secretion in perfused rat pancreas (Bertrand et al., 1991), in isolated rat islet (Petit et al., 1998), in cell lines (Kindmark et al., 1991) and in isolated human islets (Fernandez-Alvarez et al., 2001; Petit et al., 1998). In addition, the ATP analogue, ADPβS has been shown to induce vasoconstriction or vasodilatation in the pancreatic vascular bed, depending on the mediation by P2X or P2Y receptors. Extracellular ATP and UTP are agonists that act on the pancreatic duct to induce chloride secretion (Chan et al., 1996; Christoffersen et al., 1998; Hede et al., 1999; Nguyen et al., 2001).

In the present study, we have identified the P2Y and P2X purinergic receptor subtypes that are expressed in the rat pancreas during STZ-induced diabetes. Our data show an increase in $P2X_7$-positive cells in the islet of diabetic animals. The macrophage is a well-known scavenger cell and it has long been known that during diabetes, macrophages and other immune cells infiltrate into the islets (Like and Rossini, 1976; Fraser et al., 1997; Takamura et al., 1999; Jansen et al., 1994). The expression of $P2X_7$ has been described in macrophages (Coutinho-Silva and Persechini, 1997; Coutinho-Silva et al., 2001b) and inflammatory conditions can induce the up-regulation of $P2X_7$ in macrophages (Blanchard et al., 1991; Hickman et al., 1994). These observations prompted us to investigate whether STZ treatment can induce up-regulation of $P2X_7$ receptors on macrophages. Furthermore, we were interested whether

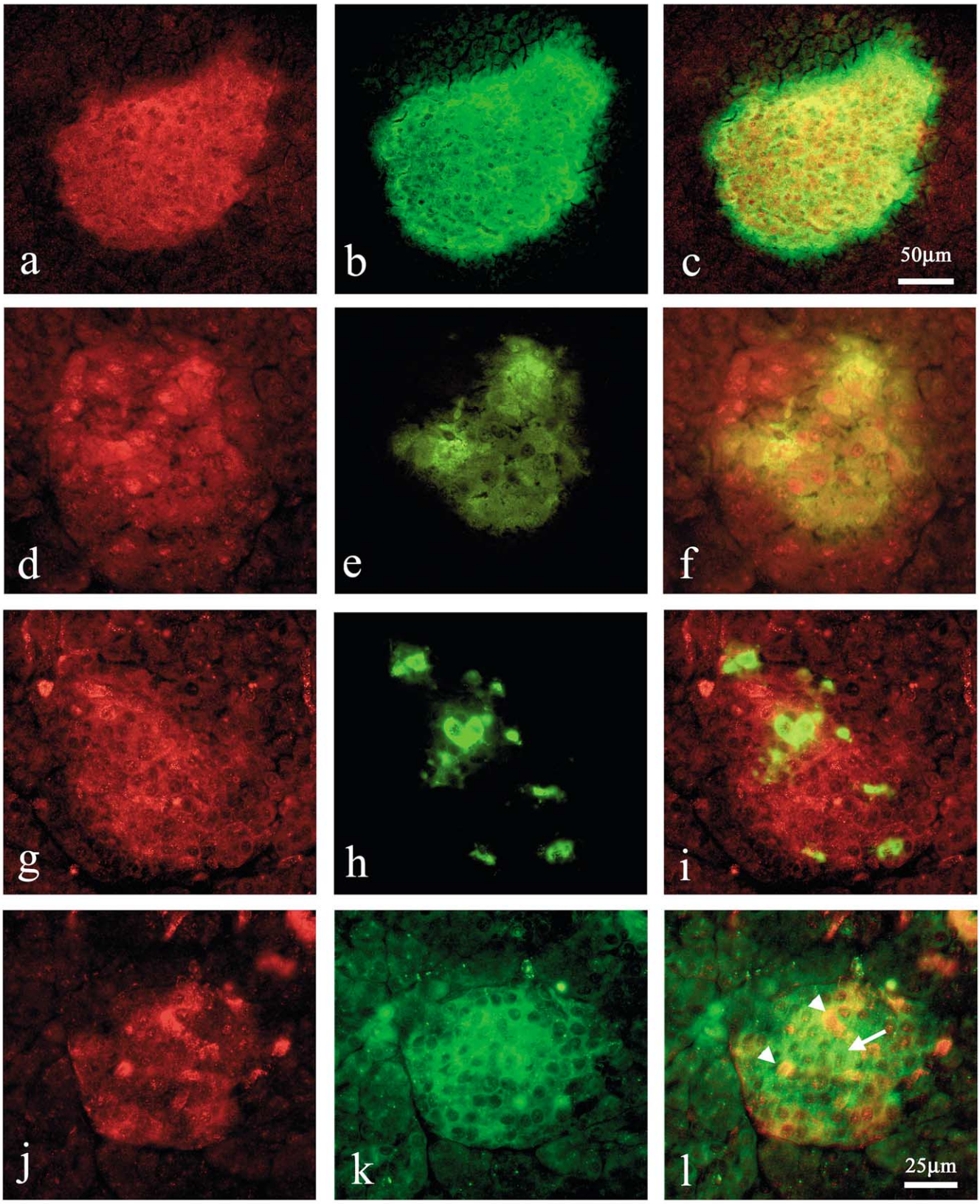


Fig. 5

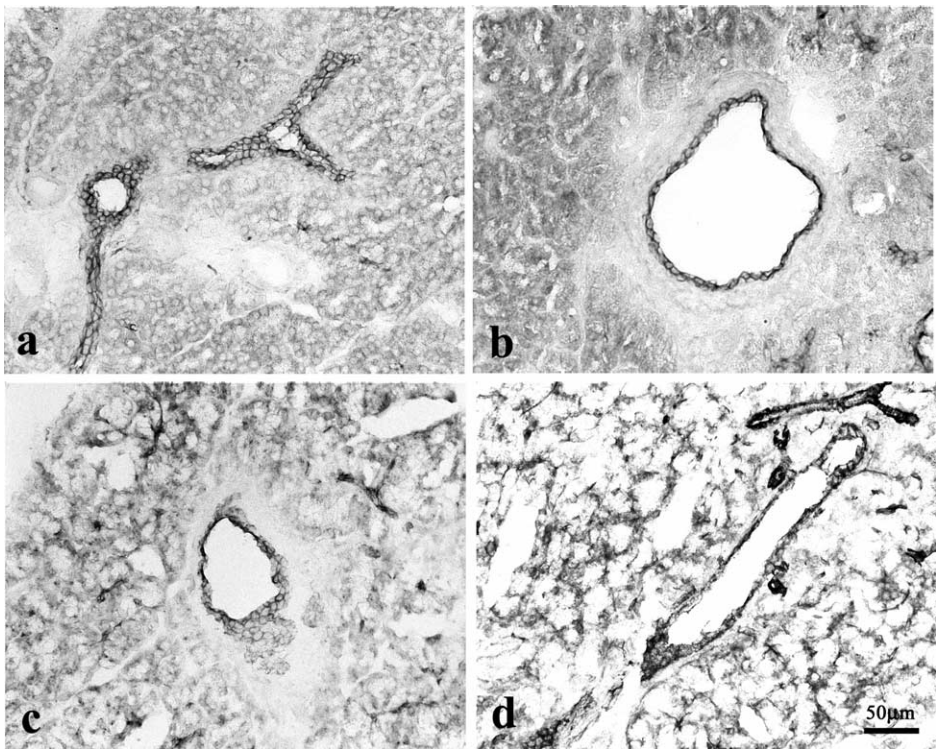


Fig. 6. P2Y receptors in pancreatic duct cells. (a) $P2Y_2$ receptor staining in cells from small ducts in normal rat. (b) $P2Y_2$ receptor staining in cells from large ducts in normal rat pancreas. (c) $P2Y_2$ receptor immunostaining in cells from medium-sized duct from 8-week diabetic rat pancreas. (d) $P2Y_1$ receptor immunostaining in duct cells from 8-week diabetic rat pancreas. Scale bar applies to all panels.

macrophages with high levels of $P2X_7$ receptor expression could be responsible for the changes observed in islets of Langerhans. However, our study showed that there was no up-regulation of $P2X_7$ receptor expression in the infiltrating macrophages in the islets from 8-week diabetic animals. Indeed, $P2X_7$ expression appeared to be lower in these macrophages. In contrast, a clear up-regulation in peritoneal and spleen macrophages was observed shortly after STZ treatment. The down-regulation of $P2X_7$ receptors in macrophages may have a functional significance in the long-term. It is known that the activation of $P2X_7$ receptors in macrophages can be cytotoxic for this cell type (Coutinho-Silva et al., 2001b) and necrotic cells are observed in diabetic islets (Bolaffi et al., 1986; Like and Rossini, 1981). It is therefore possible that ATP released could activate lysis of macrophages if they have high expression of $P2X_7$ receptors.

Double-labelling experiments showed that cells expressing high levels of $P2X_7$ receptors in the body of the islet of Langerhans are in fact α cells. This data is in agreement with our recent finding that α cells are the islet cell population that are positive for $P2X_7$ receptors in adult and ageing rat (Coutinho-Silva et al., 2001a). This is the first study reporting a change in the distribution of $P2X_7$ receptors on α cells, in a pathological condition.

In diabetic animals, we observed an increase in $P2X_7$-positive α cells, which had migrated to the centres of pancreatic islets. This is consistent with an increased number of α cells observed in a study of STZ-induced diabetes in monkey (Jones et al., 1980) and in immature mice (Riley et al., 1981). A decrease in β cells and an increase in α cells has also been shown in transgenic mice lacking ATP-sensitive K^+ channels (Miki et al., 2001, 1997). This suggests that either direct interaction by cell-

Fig. 5. Islet of Langerhans double-labelling with $P2Y_4$ receptor and insulin, somatostatin and glucagon in normal (a–c) and 12 week diabetic animals (d–l). (a) $P2Y_4$ receptor immunostaining in islet cells. (b) Insulin immunostaining showing β cells. (c) Shows (a) and (b) co-localization showing marked co-labelling (yellow) between the $P2Y_4$ receptor and insulin in many of the central cells and weak double-labelling in the periphery of the islet, in normal animals. Scale bar in c applies also to (a) and (b). (d) $P2Y_4$ receptor immunostaining. (e) Insulin immunostaining showing β cells. (f) Shows (d) and (e) co-localization in diabetic animals. Note that there is complete double-labelling in the remaining β cells (yellow). (g) $P2Y_4$ immunostaining. (h) Somatostatin immunostaining showing δ cells. (i) Shows (g) and (h) co-localization. Note that there is no co-localization. (j) $P2Y_4$ receptor immunostaining. (k) Glucagon immunostaining showing α cells. (l) Shows co-localization of (j) and (k). Note that there is clear co-localization (yellow) between $P2Y_4$ receptors and glucagon (arrowheads). However, note that not all glucagon-positive cells are immunolabelled with $P2Y_4$ receptors (arrow). Scale bar in (l) applies to (d–l).

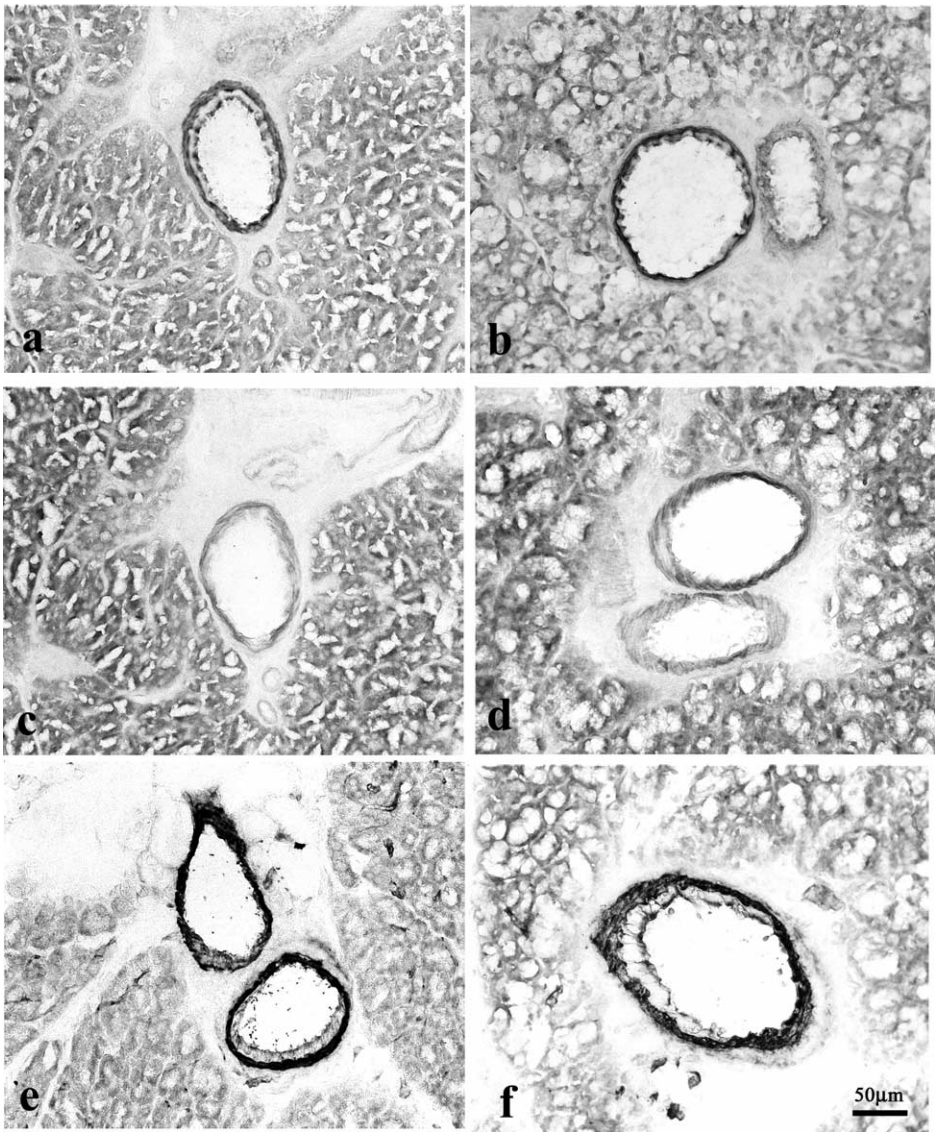


Fig. 7. P2X and P2Y receptors in pancreatic blood vessels. (a) $P2X_1$ receptor and (c) $P2X_2$ receptor staining in the vascular smooth muscle of normal rat. (b) $P2X_1$ and (d) $P2X_2$ receptor staining in vascular smooth muscle of 12-week diabetic rat. (e) $P2Y_1$ receptor immunostaining in pancreatic blood vessels from normal rat. (f) $P2Y_1$ receptor immunostaining in pancreatic blood vessels from 8-week diabetic rat. Scale bar applies to all panels.

to-cell contact or indirect interaction via unknown paracrine signals between β and α cells might be relevant for the maintenance of normal pancreatic architecture, with the number and localization of α cells under the direct influence of the β cell population.

$P2X_7$ receptors have been shown to mediate apoptosis in several different cell types including macrophages (Coutinho-Silva et al., 2001b), dendritic cells (Coutinho-Silva et al., 1999), exfoliated epithelial cells (Gröschel-Stewart et al., 1999a,b) and tumour cells (Peng et al., 1999; Janssens and Boeynaems, 2001). However, there are an increasing number of examples where the $P2X_7$ receptor is located on cells that are not undergoing apoptosis (Ugur et al., 1997; Di Virgilio et al., 1989; Humphreys et al., 1998). The presence of $P2X_7$ receptors in α cells, reported here in both healthy and diabetic pancreas, cannot at this time be clearly associated with cell death. It is possible that, as has been proposed for lymphocytes (Baricordi et al., 1996), $P2X_7$ receptors could be associated with proliferation of α cells in the pancreas. A study of transfection of $P2X_7$-deficient lymphoid cells with $P2X_7$ DNA showed that proliferation of these cells in serum-free medium is sustained and that proliferation is abolished by blocking of the $P2X_7$ receptor or ATP hydrolysis (Baricordi et al., 1999).

Recently, based on studies using a pharmacological approach and RT-PCR for mRNA, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2X_1$, $P2X_4$ and $P2X_7$ have been identified in pancreatic duct cells of the young rat (Luo et al., 1999). Our immunohistochemical data extend these findings, show-

Table 1
Summary of distribution of P2X and P2Y receptor subtypes in pancreatic tissues

	Normal rat	2 weeks diabetic	8 weeks diabetic	12 weeks diabetic
Islets				
$P2X_7$	Peripheral α cells	Peripheral and core α cells	Core α cells	Core α cells
$P2Y_4$	α and β cells	NE	α and β cells	α and β cells
$P2Y_1$	Capillaries	Capillaries	Capillaries	Capillaries
Vascular smooth muscle				
$P2X_1$	All size vessels	NE	All size vessels	All size vessels
$P2X_2$	All size vessels	NE	All size vessels	All size vessels
$P2Y_1$	All size vessels	All size vessels	All size vessels	All size vessels
$P2Y_2$	Large vessels	NE	Large vessels	Large vessels
Duct cells				
$P2Y_1$	Present	Present	Present	Present
$P2Y_2$	Present	Present	Present	Present

NE, not examined.

ing the presence of receptor protein for $P2Y_1$ and $P2Y_2$ receptors in adult rat tissue, although the expression of these two P2Y receptors was not altered in diabetic rats.

Vascular dysfunction is a typical consequence of diabetes (Kamata et al., 1989). There is a strong reduction in adenosine-induced blood vessel dilatation in the pancreas from rats with STZ-induced diabetes (Gross et al., 1989). In terms of the ATP response, it has been shown that the vasodilatory effect is preserved in the pancreatic vascular bed of 5 week, STZ-induced diabetic rats while the transient vasoconstriction observed in age-matched rats disappears (Hillaire-Buys et al., 1992). We observed the presence of $P2X_1$, $P2X_2$, $P2Y_1$ and $P2Y_2$ receptor protein in pancreatic vascular smooth muscle of diabetic animals. This may implicate a further, as yet unknown, P2X receptor in pancreatic blood vessels that is responsible for transient vasoconstriction, in addition to $P2X_1$ and $P2X_2$ receptors or it may be that there are changes in ATP release or ectoenzymatic breakdown in the diabetic pancreas.

Our present data raises several issues. Few cells in the islet of Langerhans of diabetic animals are positive for insulin (Fig. 3b). Therefore our data is not consistent with an early paper reporting that ATP-induced insulin release is the same in 5-week diabetic animals as in controls (Hillaire-Buys et al., 1992). The authors suggested that it was possible that the remaining β cells in diabetic rat pancreas were extremely sensitive to ADP-βS. The $P2Y_1$ receptor has been claimed to be responsible for ATP-stimulated insulin secretion on β cells in pharmacological experiments using rats (Fischer et al., 1999) and the inhibition of insulin secretion in mouse pancreatic β cells by ATP (Poulsen et al., 1999). Although we could not demonstrate that β cells express $P2Y_1$, immunostaining for $P2Y_1$ was strong in the intra-islet network of fenestrated blood capillaries. We have also demonstrated, for the first time, the expression of $P2Y_4$ on β cells. Thus $P2Y_4$, as well as $P2Y_2$ receptors (which we recently described on pancreas islets (Coutinho-Silva et al., 2001a)), are candidate P2 subtypes for mediating the effects of ATP in β cells.

Acknowledgements

The authors are grateful to Dave Blundell for excellent technical support and to Dr Chrystalla Orphanides for editorial assistance with the manuscript. This work was partially supported by funds from the Conselho Nacional de Desenvolvimento Cientifico e Technologico do Brasil (CNPq), the Wellcome Trust and the Juvenile Diabetes Research Foundation (Grant 1-1999-560). Dr Coutinho-Silva is a Wellcome Trust fellow, number 062754/Z00Z.

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