Expression of P2Y receptors in the rat anterior pituitary

Qiang Yu · Wei Guo · Xianmin Song · Xiaofeng Liu ·
Zhenghua Xiang · Cheng He · Geoffrey Burnstock

Received: 6 April 2011 / Accepted: 1 May 2011 / Published online: 13 May 2011
© Springer Science+Business Media B.V. 2011

Abstract In this study, the distribution patterns of P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂, and P2Y₁₃ receptors in the anterior pituitary cells of rat were studied with double-labeling immunofluorescence and Western blot. The results showed that P2Y receptors were widely expressed in the anterior pituitary. P2Y₁ and P2Y₄ receptors were found to be expressed in the majority of gonadotrophs and thyrotrophs, P2Y₂ receptors were expressed in a small subpopulation of lactotrophs and almost all the folliculo-stellate cells, that were also stained with S100 protein immunoreactivity. P2Y₆ receptors were expressed in macrophages. P2Y₁₃ receptors were expressed in a small subpopulation of cells in the rat anterior pituitary, the identity of which needs to be clarified. P2Y₁ and P2Y₄ receptors are co-expressed in some gonadotrophs and thyrotrophs. Corticotrophs and somatotrophs were found not to express P2Y receptors in this study. FSH and TSH were shown to coexist in the same endocrine cells in rat anterior pituitary. The present data suggests that purines and/or pyrimidines could be involved in regulating the functions of gonadotrophs and thyrotrophs via P2Y₁ and P2Y₄ receptors, some lactotrophs via P2Y₂

receptors, and folliculo-stellate cells via P2Y₂ receptors in the rat anterior pituitary.

Keywords P2Y receptors · Pituitary · Immunohistochemistry · Rat

Introduction

Extracellular purines and pyrimidines act as messengers via purinergic receptors on the plasma membrane. There are two purinergic receptors: P1 receptors (adenosine receptor [AR]) activated by adenosine and P2 receptors activated by ATP-, ADP-, UTP- and/or UDP. There are four AR subtypes (A₁, A_{2A}, A_{2B} and A₃), which are all G protein-coupled receptors. There are two groups of P2 receptors: P2X and P2Y receptors, which are ligand-gated ion channels and G protein-coupled receptors, respectively. So far, seven P2Y receptors and seven P2X receptors have been cloned and are denoted P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, P2Y₁₄, and P2X₁ to P2X₇ receptors [1].

There is increasing evidence to show that extracellular purines and pyrimidines, via P2 receptors, have important physiological functions in the regulation of anterior pituitary cell secretion. Using the technique of single-cell calcium measurement, the initial characterization of purinoceptors in anterior pituitary cells were identified [2–6]. These experiments revealed that functional P2X receptors are operative in all secretory cell types. With molecular biology techniques, P2X₂, P2X₃, P2X₄, and P2X₇ mRNA transcripts were detected in a mixed population of anterior pituitary cells. Lactotrophs and immortalized GH3 pituitary cells expressed transcripts for P2X₃, P2X₄, and P2X₇ subunits, somatotrophs expressed P2X₂ receptor, and

Q. Yu and W. Guo contributed equally to this work.

Q. Yu · W. Guo · X. Song · X. Liu · Z. Xiang (✉) · C. He
Department of Neurobiology, Key Laboratory of Molecular
Neurobiology, Ministry of Education,
Neuroscience Research Centre of Changzheng Hospital,
Second Military Medical University,
Shanghai 200433, China
e-mail: zhxiang@hotmail.com

G. Burnstock
Autonomic Neuroscience Centre,
University College Medical School,
Royal Free Campus, Rowland Hill Street,
London NW3 2PF, UK

thyrotrophs and corticotrophs also express P2X receptors [7]. Single-cell patch-clamp analysis in gonadotrophs from embryonic, neonatal, and adult rats revealed that P2X₂ receptors are operative and involved in release of luteinizing hormone (LH) and regulation of gonadotrophin-releasing hormone (GnRH) controlled electrical activity and secretion [8]. Lactotrophs express P2X₄ receptors, which facilitate Ca²⁺ influx and hormone secretion [9].

Compared with data for P2X receptors on the anterior pituitary cells, there is less available data for P2Y receptors on these cells. Using calcium image analysis, sheep and rat anterior pituitary cells were found to express P2Y receptors [10–12]. Following RT-PCR analysis, P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₂ receptor transcripts were detected in mixed anterior pituitary cells [13, 14]. The identified cells in the anterior pituitary with P2Y receptors were also found in rat pituitary folliculo-stellate cells in primary culture [15], gonadotrophs [16] and lactotrophs [17]. No detailed immunofluorescence, Western blot or in situ hybridization studies about P2Y receptor subtype expression on the individual cell types in the anterior pituitary are currently available.

In this study, we used single-labeling and double-labeling immunofluorescence, in situ hybridization and Western blot analyses to study the distribution patterns of P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂, and P2Y₁₃ receptors in anterior pituitary cells of rat and found that gonadotrophs and thyrotrophs express P2Y₁ and P2Y₄ receptors, lactotrophs and folliculo-stellate cells express P2Y₂, and macrophages express P2Y₆.

Materials and methods

Animals and tissue preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University. Five Sprague–Dawley rats were used. Animals were anesthetized by intraperitoneal injection with chloral hydrate and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. The pituitaries were dissected out and refixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4 for 4–6 h, then transferred to 25% sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the segment blocks were rapidly frozen and crosscut sections (10 µm in thickness) were cut with a Leica cryostat and thawed on slides covered with gelatin.

Immunohistochemistry

Table 1 shows the primary antibodies used in this study.

The following protocol is a double immunofluorescence technique for two primary antibodies from different hosts. The section slides were washed 3×5 min in PBS, and then preincubated in antiserum solution 1 (10% normal bovine serum, 0.2% Triton-X-100, 0.4% sodium azide in 0.01 mol/l PBS pH7.2) for 30 min, followed by incubation with two primary antibody dilutions, one a P2Y antibody (P2Y₆, P2Y₁₂, and P2Y₁₃) and the second either a hormonal antibody adrenocorticotropin (ACTH), follicle-stimulating hormone (FSH), prolactin (PRL), and thyroid-stimulating hormone (TSH) or a cell type marker (ED1 [macrophage marker] and S-100 [folliculo-stellate cell marker]) at room temperature, overnight. Subsequently, the sections were incubated at room temperature for 2 h with a mixed secondary antibody solution of Cy3-conjugated donkey anti-rabbit IgG diluted 1:400 for P2Y antibodies and either FITC-conjugated donkey anti-goat IgG diluted 1:200 for hormonal antibodies or FITC-conjugated donkey anti-mouse IgG diluted 1:200 for cell type marker antibodies. All the incubations and reactions were separated by 3×10 min washes in PBS.

The following protocol is a double immunofluorescence technique for two primary antibodies from the same hosts. Simultaneous detection of two antigens by immunostaining usually requires primary antibodies from two different species. A novel double-labeling immunostaining method for immunodetection of two independent antigens has been described [18]. The principle of the method is that the first antigen is detected by the first primary antibody that is diluted so extensively that it cannot be detected with conventional methods; a highly sensitive tyramide signals amplification (TSA) system is used to identify this antibody; the second antigen is stained with the secondary primary antibody and detected by conventional immunostaining. We have used this double-labeling protocol of fluorescence immunohistochemistry successfully [19, 20]. The following protocol was modified from this protocol. Endogenous peroxidase was blocked by 1% H₂O₂ in PBS for 30 min. The sections were pre-incubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by incubation with P2Y antibodies diluted in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight at 4°C. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in extravidin peroxidase (Sigma) diluted 1:1,000 in PBS for 30 min at room temperature. P2Y immunoreactivity was visualized by the TSA Fluorescein system (NEL701, NEN, USA). After visualization the sections were incubated with the second primary antibody of either a hormone or a cell type marker, diluted in the antiserum

Table 1 Primary antibodies used in this study

Primary antibodies	Host	Manufacturer	Catalog no.	Dilution	
				IHC	TSA
P2Y ₁	Rabbit	Alomone	APR-009	1:200	1:4,000
P2Y ₂	Rabbit	Alomone	APR-010	1:400	1:8,000
P2Y ₄	Rabbit	Alomone	APR-006	1:400	1:8,000
P2Y ₆	Goat	Santa Cruz	SC-15215	1:100	
P2Y ₁₂	Goat	Santa Cruz	SC-27152	1:50	
P2Y ₁₃	Goat	Santa Cruz	SC-69526	1:100	
ACTH	Rabbit	Sigma	A1927	1:600	1:10,000
FSH	Rabbit	Chemicon	AB928	1:600	1:10,000
PRL	Rabbit	Chemicon	AB960	1:200	1:5,000
TSH	Rabbit	Chemicon	AB976	1:400	1:10,000
S-100	Mouse	Abcam	ab4066	1:200	
ED1	Mouse	Abcam	ab31630	1:200	
Digoxigenin	Mouse	Jackson	200-002-156	1:200	

dilution solution overnight at 4°C. Subsequently, the sections were incubated with Cy3 conjugated donkey–anti–rabbit or mouse (Jackson ImmunoResearch) diluted 1:400 in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3×10 min washes in PBS. Some sections were counter-stained with 5 µg/ml Hoechst 33342.

Combined use of in situ hybridization and immunofluorescence

As the proper antibody for growth hormone could not be obtained for this study, sense and antisense digoxigenin-labeled oligonucleotide probes were synthesised (Sagon, Shanghai, China). The sense and antisense nucleotide sequences are AGGGCATCCAGGCTCTGATGCAG GAGCTGG, CCAGCTCCTGCATCAGAGC CTGGATGCCCT (GenBank Accession: U62779.1), respectively. In situ hybridization was carried out as following. The sections were heated in 0.01 mol/l citrate buffer pH6.8 in a 92°C water bath for 15 min and cool to room temperature in the same buffer. The sections were rinsed in PBS for 3 min for two times, then incubated in 0.25% acetic anhydride with 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature, followed by washing in 2× saline sodium citrate (SSC) for 10 min. Digoxigenin-labeled oligonucleotides (0.5 µg/ml) of either antisense or sense probe was added to the hybridization solution containing 50% formamide, 10% dextrans sulphate, 0.3 M NaCl, 1× Denhardt's solution, 0.05 M Tris–HCl (pH 8.0), 1 mM EDTA and 250 mg/ml Herring sperm DNA (Sigma). Hybridization was carried out for 12–16 h at 37°C in a hybridization oven. The sections were washed in 4× SSC

for 20 min, in 2× SSC for 20 min, in 1× SSC for 20 min, in 0.5× SSC for 20 min at 37°C.

The following method was used to detect hybridization signals and P2Y receptors. Endogenous peroxidase was blocked by 3% H₂O₂ in PBS for 30 min. The sections were pre-incubated in 10% NHS, 0.2% Triton X-100 in PBS for 30 min, followed by incubation with two primary antibodies (mouse anti-digoxigenin IgG and one of the P2Y antibodies) diluted in antibody dilution solution overnight at 4°C. Subsequently, the sections were incubated with two secondary antibodies of biotinylated donkey anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) at a dilution of 1:500 and 1:200, respectively, in PBS containing 1% NHS for 1 h. The sections were then incubated in extravidin peroxidase (Sigma) diluted 1:1,000 in PBS for 30 min at room temperature. The digoxigenin immunoreactivity was visualized by the TSA Cy3 system (NEL704A, NEN life science, USA). All the incubations and reactions were separated by 3×10 min washes in PBS. Some sections were counter-stained with 5 µg/ml Hoechst 33342.

Western blot

Sprague–Dawley rats were deeply anesthetized by sodium pentobarbital (60 mg/kg) and killed by decapitation. The pituitaries were rapidly removed and lysed with 20 mM Tris–HCl buffer, pH 8.0, containing 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma). Protein concentration was determined by the BCA protein assay method using bovine serum albumin as standard (BCA protein assay kit from

Beyotime). Protein samples (100 µg) were loaded in each lane, separated by SDS-PAGE (10% polyacrylamide gels) and then electrotransferred onto nitrocellulose membranes. The membranes were blocked with 10% non-fat dry milk in Tris-buffered saline for 1 h and incubated overnight at 4°C with the P2Y antibodies diluted 1:200–400 in PBS. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-Goat or Rabbit IgG (Beyotime) diluted 1:1,000 in 2% BSA in PBS for 1 h at room temperature. The color development was performed with 400 µg/ml nitro-blue tetrazolium, 200 µg/ml 5-bromo-4-chloro-3-indolyl phosphate and 100 mg/ml levamisole in TSM2 (0.1 mol/l Tris-HCl₂ buffer, pH 9.5, 0.1 mol/l NaCl and 0.05 mol/l MgCl₂) in the dark. Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories).

Photomicroscopy

Images were taken with the Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop). The two-channel readings for green and red fluorescence were merged by using Adobe Photoshop. The focal plane on the microscope was not adjusted whilst determining whether a particular cell co-localized both P2Y receptors and other markers. Only cells that demonstrated the same morphology, orientation and position when viewed under the two different filters (in the same focal plane) for the detection of Cy3 and FITC were deemed to co-localize both P2Y receptor and other markers.

Quantitative analysis

Quantitative analysis for the P2Y receptors immunostaining in the anterior pituitary was performed as follows: five random fields (each area was 0.36 mm²) for one section were chosen and the number of positive cells was counted and expressed as the positive cell numbers per square millimeter. Five fields for each of five sections from each of five rats were used. The mean number of positive cells/mm² from each rat was calculated and data are expressed as the mean ± standard error of the mean (n = number of rat).

Results

With double-labeling immunofluorescence and combined in situ hybridization and immunofluorescence, P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₃ receptors were found to be expressed in the different cell types of the rat anterior pituitary. Control experiments and Western blot confirmed the specificity of

P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂, P2Y₁₃ receptor immunoreactivity in the anterior pituitary cells of rat.

P2Y₁ receptor-immunoreactive (–ir) cells were generally ovoid in shape with moderate to strong immunostaining, some of P2Y₁ receptor-ir cells were angular in shape with processes (Fig. 1). Double-labeling immunofluorescence showed that 90% and 93% of P2Y₁ receptor-ir cells were also immunoreactive for FSH and TSH, respectively, but none of them were immunoreactive for ACTH, PRL or S100 in the anterior pituitary of rat (Fig. 1). There were still about 32% of FSH-ir endocrine cells and 35% of TSH-ir endocrine cells, which were not found to express P2Y₁ receptors. Growth hormone (GH) mRNA hybridization signals were also not detected in the P2Y₁ receptor-ir cells in the anterior pituitary (Fig. 1c). P2Y₂ receptor-ir cells were distributed widely in the rat anterior pituitary, and some of the positive cells with long processes were strongly immunostained and angular in shape (Fig. 2). Double-labeling immunofluorescence showed that about 38% of P2Y₂ receptor-ir cells were also immunoreactive for PRL, but none of them were immunoreactive for ACTH, FSH, or TSH. GH mRNA hybridization signals were also not detected in the P2Y₂ receptor-ir cells in the anterior pituitary (Fig. 2c). About 61% of P2Y₂ receptor-ir cells with strong immuno-labeling were also labeled by the S100 antibody in the anterior pituitary. Almost all of the S100-ir cells were also labeled by the P2Y₂ antibody (Fig. 2f). The distribution pattern of P2Y₄ receptor-ir cells was very similar with that of the P2Y₁ receptor-ir cells. P2Y₄ receptor-ir cells were also generally ovoid in shape with strong immunostaining (Fig. 3). Double-labeling immunofluorescence showed that almost all of the P2Y₄ receptor-ir cells were also labeled by FSH or TSH antibodies, but none of them were labeled by ACTH, GH, PRL or S100 antibodies (Fig. 3a, c, d, f). There were about 29% FSH-ir endocrine cells and 36% TSH-ir endocrine cells, which were found not to express P2Y₄ receptors, respectively (Fig. 3b, e). GH mRNA hybridization signals were not detected in the P2Y₄ receptor-ir cells in the anterior pituitary (Fig. 3c). P2Y₆ receptor-ir cells were also found in the rat anterior pituitary, but they were very small in size and scattered in the anterior pituitary. These positive cells were not labeled by ACTH, FSH, PRL, TSH or S100 antibodies, or GH oligonucleotide probes, but were labeled by ED1 (a macrophage marker) (Fig. 4a, b, c). No P2Y₁₂ receptor-ir cells were detected in the rat pituitary (data not showed). P2Y₁₃ receptor-ir cells were detected in the anterior and intermediate lobes of the rat pituitary. In the anterior lobe, the number of P2Y₁₃ receptor-ir cells was relatively low. They were found in clusters formed by a few positive cells. These P2Y₁₃ receptor-ir cells were not labeled by ACTH, FSH, PRL, TSH, ED1 or S100 antibodies or GH oligonucleotide probes. P2Y₁₃ receptor-

Fig. 1 Expression of P2Y₁ receptors and co-localization with pituitary hormones in the anterior pituitary of the rat. **a** Merged image of P2Y₁ receptor-immunoreactive (ir) (red) and ACTH-ir (green) cells. Note that there are no colocalisation (yellow) of P2Y₁ receptor- and ACTH-ir cells. **b** Merged image of P2Y₁ receptor-ir (red) and FSH-ir cells (green). Note that many P2Y₁ receptor-ir cells are labeled with FSH immunoreactivity. Colocalised cells are yellow/orange in color, such as the one indicated by a yellow arrow. Single-labeled FSH-ir and P2Y₁-ir cells are also observed. A pink arrow and a blue arrow each indicate a single labeled FSH-ir and P2Y₁-ir cell, respectively. **c** Merged image of P2Y₁ receptor-ir (green) and GH mRNA (red) cells. Note that there is no colocalisation of P2Y₁ receptor-ir and GH

mRNA stained cells. **d** Merged image of P2Y₁ receptor-ir (red) and PRL-ir cells (green). Note that there is no colocalisation of P2Y₁ receptor- and PRL-immunoreactive cells. **e** Merge image of P2Y₁ receptor-ir (red) and TSH-ir (green) cells. Note that many P2Y₁ receptor-ir cells are also labeled with TSH-immunoreactivity. Double-labeled cells are yellow in color, such as the one indicated by a yellow arrow. Single labeled FSH-ir and P2Y₁-ir cells are also observed. A pink arrow and a blue arrow each indicate a single labeled FSH-ir and P2Y₁-ir cell, respectively. **f** Merged image of P2Y₁ receptor-ir (red) and S100-ir (green) cells. Note that there were no cells doubled labeled (yellow) with P2Y₁ receptor- and S100- immunoreactivity. All scale bars=60 μ m

ir cells were also found in the intermediate lobe of the rat pituitary and these P2Y₁₃ receptor-ir cells were also labeled by the ACTH antibody (Fig. 4d, e, f). Of the P2Y receptor family members examined in this study, only the P2Y₁₃ receptor was found to be expressed in the intermediate lobe

of the rat pituitary. Table 2 summarizes the expression of P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₃ receptors in the different cell types of the rat anterior pituitary cells. Tables 3, 4 and 5 summarize the data of the quantitative analysis of expressions of P2Y₁, P2Y₂, and P2Y₄ receptors

Fig. 2 Expression of P2Y₂ receptors and co-localization with pituitary hormones in the anterior pituitary of the rat. **a** Merged image of P2Y₂ receptor-immunoreactive (ir) (red) and ACTH-ir (green) cells. Note that there was no colocalisation between P2Y₂ receptor- and ACTH-ir cells. **b** Merged image of P2Y₂ receptor-ir (red) and FSH-ir cells (green). Note that there was no colocalisation between P2Y₂ receptor- and FSH-ir cells. **c** Merged image of P2Y₂ receptor-ir (green) and GH mRNA stained (red) cells. Note that no colocalised cells with P2Y₂ receptors and GH mRNA signals are observed. **d** Merged image of P2Y₂ receptor-ir (red) and PRL-ir cells (green). Note that some P2Y₂ receptor-ir cells are also immunoreactive for PRL.

One of the double-labeled cells (yellow in color) is indicated by a yellow arrow. Single-labeled FSH-ir and P2Y₂-ir cells are also observed. A pink arrow and a blue arrow indicate a single labeled FSH-ir and P2Y₂-ir cell, respectively. **e** Merged image of P2Y₂-ir and TSH-ir cells. Note that there is no colocalisation. **f** a merged image of P2Y₂ receptor-ir (red) and S100-ir (green) cells. Note that almost all of the S100-ir cells are also labeled with P2Y₂ immunoreactivity, but a proportion of the P2Y₂-ir cells are not labeled by S100 antibody, such as the one indicated by a red arrow. A yellow arrow indicates a double-labeled cell. All scale bars=60 μm

in gonadotrophs, lactotrophs, thyrotrophs and folliculostellate cells in the anterior pituitary. As P2Y₁ and P2Y₄ receptors were found to coexist with FSH or TSH, it implies that P2Y₁ and P2Y₄ receptors, and FSH and TSH may coexist in the same endocrine cells. As such, double-labeling immunostaining experiments were carried out to

clarify this issue. The results showed that almost all of the P2Y₁ receptor-ir cells were also labeled by the P2Y₄ receptor antibody and vice versa (Fig. 5a, b, c); in addition, almost all of the FSH-ir cells were also labeled by the TSH antibody, and vice versa (Fig. 5d, e, f). Tables 6 and 7 summarize the data of the coexistence of P2Y₁ and P2Y₄

Fig. 3 Expression of P2Y₄ receptors and co-localization with pituitary hormones in the anterior pituitary of the rat. **a** Merged image of P2Y₄ receptor-ir (red) and ACTH-ir (green) cells. Note that no cells double-labeled (yellow) with P2Y₄ receptor- and ACTH-immunoreactivity are observed. **b** Merged image of P2Y₄ receptor-ir (red) and FSH-ir cells (green). Note that almost all P2Y₄ receptor-cells are labeled with FSH immunoreactivity. Double-labeled cells are yellow in color, such as the one indicated by a yellow arrow. Single-labeled FSH-ir cells are also observed, such as the one indicated by a pink arrow. **c** Merged image of P2Y₄ receptor-ir (green) and GH mRNA stained (red) cells. Note that there are no cells double labeled

for P2Y₄ receptors and GH mRNA signals. **d** Merged image of P2Y₄ receptor-ir (red) and PRL-ir cells (green). Note that there are no cells double labeled with P2Y₄ receptor- and PRL-immunoreactivity. **e** Merge image of P2Y₄ receptor-ir (red) and TSH-ir (green) cells. Note that all P2Y₄ receptor-ir cells are also labeled with TSH immunoreactivity. Double-labeled cells are yellow in color, such as the one indicated by a yellow arrow. Single-labeled FSH-ir cells are also observed, such as the one indicated by a pink arrow. **f** Merged image of P2Y₄ receptor-ir (red) and S100-ir (green) cells. Note that there are no cells double labeled (yellow) with P2Y₄ receptor- and S100 immunoreactivity. All scale bars=60 μ m

receptors, and FSH and TSH in endocrine cells of the rat anterior pituitary, respectively.

Western blotting, performed on tissue extracts derived from the rat pituitary (Fig. 6a), assessed the specificity of the polyclonal P2Y receptor antibodies. The immunoreactive bands were detected at different molecular weight

levels: one band for the P2Y₁ receptor at about 48 kDa (Fig. 6a, lane 1), two bands for the P2Y₂ receptor at about 36, 90 kDa (Fig. 6a, lane 3), one band for the P2Y₄ at about 49 kDa (Fig. 6a, lane 5), one band for the P2Y₁₃ receptor at about 37 kDa (Fig. 6a, lane 9), but no clear bands for P2Y₆ and P2Y₁₂ receptors were found (Fig. 6a, lanes 7 and 8).

Fig. 4 Expression of P2Y₆ and P2Y₁₃ receptors in the rat pituitary. **a** Expression of P2Y₆ receptors in the anterior pituitary. Note that the positive cells are small in size. **b** ED1 expression in the same area of **a**. **c** Merged image of **a** and **b**. Note that all the P2Y₆ receptor-ir cells are also labeled with ED1 immunoreactivity, such as the one indicated by a *yellow arrow*. **d** Expression of P2Y₁₃ receptors in the intermediate lobe of the rat pituitary. Note that numerous positive

cells are detected in the intermediate lobe, while there are only scattered positive cells in the anterior lobe. **e** ACTH expression in the same area of **d**. **f** Merged image of **d** and **e**. Note that almost all of the P2Y₁₃ receptor-ir cells in the intermediate lobe are also labeled with ACTH immunoreactivity, such as the one indicated by a *yellow arrow*, but not labeled with ACTH immunoreactivity in the anterior lobe, such as the one indicated by a *pink arrow*. All scale bars=60 μm

Preadsorption of the antisera with the peptide antigens resulted in the absence of the bands (Fig. 6a, lanes 2, 4, 6 and 10), indicating that the antibodies detected the appropriate antigen sequences.

Control experiments were carried out with the P2Y antibodies preabsorbed with their own peptides. No staining was observed in those preparations incubated with antisera solutions preabsorbed with their own peptides, as shown for

Table 2 Coexistence between P2Y receptors and pituitary hormones or S100

P2Y	ACTH	FSH	GH	PRL	TSH	S100
P2Y ₁	–	+	–	–	+	–
P2Y ₂	–	–	–	+	–	+
P2Y ₄	–	+	–	–	+	–
P2Y ₆	–	–	–	–	–	–
P2Y ₁₂	–	–	–	–	–	–
P2Y ₁₃	–	–	–	–	–	–

+ coexistence, – no coexistence

the P2Y₁ and P2Y₂ receptors (Fig. 6b, c). A further negative control, of omitting the primary antibodies, was also carried out. No staining was also observed in those preparations.

Discussion

The present study showed that corticotroph, gonadotroph, somatotroph, lactotroph, thyrotroph and follicle-stellate cells

Table 3 Quantitative analysis of the coexistence between P2Y₁ receptor-ir cells and FSH, PRL, TSH and S100 (the positive cell number/0.36 mm²)

	P2Y ₁ ⁺		P2Y ₁ [–]	
	Number	%	Number	%
FSH ⁺	46±15	90 (p1)	22±8	32 (p9)
FSH [–]	5±3	10 (p2)	NA	NA
PRL ⁺	0	0 (p3)	52±15	100 (p10)
PRL [–]	43±10	100 (p4)	NA	NA
TSH ⁺	52±16	93 (p5)	28±10	35 (p11)
TSH [–]	4±2	7 (p6)	NA	NA
S100 ⁺	0	0 (p7)	36±9	100 (p12)
S100 [–]	42±12	100 (p8)	NA	NA

P2Y₁⁺ FSH⁺, P2Y₁ receptor-ir cells also expressing FSH; P2Y₁⁺ FSH[–], P2Y₁ receptor-ir cells not expressing FSH; P2Y₁[–] FSH⁺, FSH-ir cells not expressing P2Y₁; P2Y₁[–] FSH[–], cells expressing neither P2Y₁ receptors nor FSH; NA, not assayed; p1=number of P2Y₁⁺ FSH⁺/total number of P2Y₁⁺ cells×100; p2=the number of P2Y₁⁺ FSH[–]/total number of P2Y₁⁺ cells×100; p3=the number of P2Y₁[–] PRL⁺/total number of P2Y₁[–] cells×100; p4=number of P2Y₁[–] PRL[–]/total number of P2Y₁[–] cells×100; p5=number of P2Y₁⁺ TSH⁺/total number of P2Y₁⁺ cells×100; p6=number of P2Y₁[–] TSH⁺/total number of P2Y₁[–] cells×100; p7=number of P2Y₁⁺ S100⁺/total number of P2Y₁⁺ cells×100; p8=the number of P2Y₁[–] S100⁺/total number of P2Y₁[–] cells×100; p9=number of FSH⁺ P2Y₁[–]/total number of FSH⁺ cells×100; p10=number of PRL⁺ P2Y₁[–]/total number of PRL⁺ cells×100; p11=number of P2Y₁[–] TSH⁺ cells/total number of TSH⁺ cells×100; p12=number of P2Y₁[–] S100⁺ cells/total number of S100⁺ cells×100

Table 4 Quantitative analysis of the coexistence of P2Y₂ receptor-ir cells and FSH, PRL, TSH and S100 (positive cell number/0.36 mm²)

	P2Y ₂ ⁺		P2Y ₂ [–]	
	Number	%	Number	%
FSH ⁺	0	0 (p1)	73±25	100 (p9)
FSH [–]	63±21	100 (p2)	NA	NA
PRL ⁺	21±8	38 (p3)	37±11	64 (p10)
PRL [–]	34±10	62 (p4)	NA	NA
TSH ⁺	0	0 (p5)	75±26	100 (p11)
TSH [–]	59±19	100 (p6)	NA	NA
S100 ⁺	38±15	61 (p7)	0	0 (p12)
S100 [–]	24±7	39 (p8)	NA	NA

P2Y₂⁺ FSH⁺, P2Y₂ receptor-ir cells also expressing FSH; P2Y₂⁺ FSH[–], P2Y₂ receptor-ir cells not expressing FSH; P2Y₂[–] FSH⁺, FSH-ir cells not expressing P2Y₂; P2Y₂[–] FSH[–], cells expressing neither P2Y₂ receptor nor FSH; NA, not assayed; p1=number of P2Y₂⁺ FSH⁺/total number of P2Y₂⁺ cells×100; p2=number of P2Y₂⁺ FSH[–]/total number of P2Y₂⁺ cells×100; p3=number of P2Y₂[–] PRL⁺/total number of P2Y₂[–] cells×100; p4=the number of P2Y₂[–] PRL[–]/total number of P2Y₂[–] cells×100; p5=number of P2Y₂⁺ TSH⁺/total number of P2Y₂⁺ cells×100; p6=number of P2Y₂[–] TSH⁺/total number of P2Y₂[–] cells×100; p7=number of P2Y₂⁺ S100⁺/total number of P2Y₂⁺ cells×100; p8=number of P2Y₂[–] S100⁺/total number of P2Y₂[–] cells×100; p9=number of FSH⁺ P2Y₂[–]/total number of FSH⁺ cells×100; p10=number of PRL⁺ P2Y₂[–]/total number of PRL⁺ cells×100; p11=number of P2Y₂[–] TSH⁺ cells/total number of TSH⁺ cells×100; p12=number of P2Y₂[–] S100⁺/total number of S100⁺ cells×100

in the anterior pituitary express at least one of the P2Y receptor family: the corticotroph, somatotroph, lactotroph and follicle-stellate cells express P2Y₂ receptors; gonadotroph and thyrotroph express P2Y₁, P2Y₂ and P2Y₄ receptors. The identity of a small subpopulation of cells with P2Y₁₃ receptors in the rat anterior pituitary is confirmed in this study. The results presented in this study are credible as strict control experiments were carried out. Firstly, no immunostaining in the pituitary sections was obtained in the control experiments where the P2Y antibodies were preabsorbed with their corresponding peptides or by omitting the P2Y antibodies. Secondly, preadsorption of the P2Y receptor antisera with their corresponding peptide antigens resulted in the absence of bands, indicating that the antibody detects the appropriate antigen sequence, further confirming the specificity of these P2Y receptors.

There are eight receptors of the P2Y family that have been cloned, namely P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄, all of which are G protein-coupled receptors. There are two subgroups of G proteins which P2Y receptors are coupled with, namely G_q and G_i. P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptors are linked to activation of phospholipase C (PLC), inositol lipid signalling and the mobilization of intracellular Ca²⁺, P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors are coupled with G_i protein

Table 5 Quantitative analysis of the coexistence between P2Y₄ receptor-ir cells and FSH, PRL, TSH and S100 (positive cell number/0.36 mm²)

	P2Y ₄ ⁺		P2Y ₄ ⁻	
	Number	%	Number	%
FSH ⁺	52±17	95 (p1)	21±7	29 (p9)
FSH ⁻	3±2	5 (p2)	NA	NA
PRL ⁺	0	0 (p3)	47±15	100 (p10)
PRL ⁻	62±18	100 (p4)	NA	NA
TSH ⁺	56±21	97 (p5)	31±14	36 (p11)
TSH ⁻	2±1	3 (p6)	NA	NA
S100 ⁺	0	0 (p7)	42±12	100 (p12)
S100 ⁻	49±22	100 (p8)	NA	NA

P2Y₄⁺FSH⁺, P2Y₄ receptor-ir cells also expressing FSH; P2Y₄⁺FSH⁻, P2Y₄ receptor-ir cells not expressing FSH; P2Y₄⁻FSH⁺, FSH-ir cells not expressing P2Y₄; P2Y₄⁻FSH⁻, expressing neither P2Y₄ receptor-ir nor FSH-ir cells; NA, not assayed; p1=number of P2Y₄⁺FSH⁺/total number of P2Y₄⁺ cells×100; p2=number of P2Y₄⁺FSH⁻/total number of P2Y₄⁺ cells×100; p3=number of P2Y₄⁺PRL⁺/total number of P2Y₄⁺ cells×100; p4=number of P2Y₄⁺PRL⁻/total number of P2Y₄⁺ cells×100; p5=number of P2Y₄⁺TSH⁺/total number of P2Y₄⁺ cells×100; p6=number of P2Y₄⁺TSH⁻/total number of P2Y₄⁺ cells×100; p7=number of P2Y₄⁺S100⁺/total number of P2Y₄⁺ cells×100; p8=number of P2Y₄⁺S100⁻/total number of P2Y₄⁺ cells×100; p9=number of FSH⁺ P2Y₄⁻/total number of TSH⁺ cells×100; p10=number of PRL⁺ P2Y₄⁻/total number of PRL⁺ cells×100; p11=number of P2Y₄⁻TSH⁺ cells/total number of TSH⁺ cells×100; p12=number of P2Y₄⁻S100⁺ cells/total number of S100⁺ cells×100

resulting in inhibition of cAMP formation and P2Y₁₁ (only in human) are coupled with G_s and G_q, resulting in increased cAMP [21]. Within the family of P2Y receptors, P2Y₁, P2Y₁₂ and P2Y₁₃ receptors respond to ADP. P2Y₂ and P2Y₄ receptors respond to both ATP and UTP, and P2Y₆ receptors to UDP [1]. One question must be asked, where are the endogenous purines (ATP and ADP) and pyrimidines (UTP and UDP) released from in the anterior pituitary? Currently, the physiological sources of extracellular purines and pyrimidines required for activation of purinergic receptors in pituitary cells remains unknown.

In general, neurons, neuroendocrine cells and platelets release ATP by Ca²⁺-controlled exocytosis of nucleotides stored within synaptic vesicles or dense core granules. The magnocellular neuroendocrine cells in the hypothalamus, that control release of vasopressin and oxytocin, also contain secretory ATP and could control the release of ATP [22]. Recently, the data was presented showing that ATP is also released by normal and immortalized anterior pituitary cells at resting conditions. Such basal ATP release was enhanced in cells simulated by ARL67156, an inhibitor of ectonucleotidases [13]. GnRH-induced stimulation of gonadotropin release was accompanied by elevation in basal ATP release, raising the possibility that ATP is stored

in the secretory vesicles of these cells [2]. This is consistent with an earlier study showing calcium dependence of ATP release [23]. The roles of ATP as a neurotransmitter or co-transmitter are well established in the peripheral and central nervous systems [24]. Several reports have also suggested that ATP is secreted by skeletal muscle, adrenal chromaffin cells, mast cells, blood cells, fibroblasts and endothelial cells, pyrimidines are secreted by endothelial, epithelial and astrocytoma cells [24, 25]. The duration and distance of ATP actions are limited by several ecto-ATPases, which ensure that circulating levels of ATP are below that required for the global activation of purinceptors [7]. These previous data suggested that purines and pyrimidines could be released by the anterior pituitary cells and act as an autocrine/paracrine factor to be involved in the regulation of physiological functions of anterior pituitary endocrine cells.

Rat gonadotrophs show a potency order of ATP=ADP=UTP, suggesting the presence of P2Y₁, P2Y₁₂, P2Y₁₃ (ADP) and P2Y₂ and P2Y₄ (ATP=UTP) receptors [26]. UTP-sensitive receptors have been suggested to be present in gonadotrophs [16, 26] and lactotrophs [17], but these results were not confirmed by other reports [2, 14]. The present results showed that gonadotrophs and lactotrophs express P2Y₄ and P2Y₂ receptors, respectively, the ligand for which is UTP, confirming that gonadotrophs and lactotrophs express UTP-sensitive receptors.

In this study, 91% and 95% of P2Y₁ receptor-ir cells in the rat anterior pituitary were labeled with FSH and TSH immunoreactivity, respectively. Almost all the P2Y₄ receptor-ir cells were labeled with FSH and TSH immunoreactivity. This result implies that at least a subpopulation of gonadotrophs and thyrotrophs co-express FSH and TSH in the rat anterior pituitary. In order to confirm this, we studied the coexistence of FSH and TSH in the anterior pituitary and found that almost all the gonadotrophs labeled by the FSH antibody were also labeled by the TSH antibody, which means that FSH and TSH coexist in the same endocrine cells in the anterior pituitary. A report showing that FSH and TSH coexist in the same endocrine cells of the musk shrew anterior pituitary has been published [27]. To our knowledge, this study is the first report that FSH and TSH coexist in the same endocrine cells of the rat pituitary. As the majority of gonadotrophs or thyrotrophs were found to express P2Y₁ or P2Y₄ receptors, this implies that P2Y₁ and P2Y₄ receptors may be co-expressed in a subpopulation of gonadotrophs and thyrotrophs. Further, double-labeling immunostaining confirmed that P2Y₁ and P2Y₄ receptors actually coexpress in the same endocrine cells in the anterior pituitary of the rat. As the endogenous ligands for P2Y₁ and P2Y₄ receptors are ADP and UTP, purines and pyrimidines may act on gonadotrophs and thyrotrophs at the same time if P2Y₁

Fig. 5 Colocalization of P2Y₁ and P2Y₄ receptors, and FSH and TSH in the rat anterior pituitary. **a** Expression of P2Y₁ receptors in the anterior pituitary. **b** P2Y₄ expression in the same area of **a**. **c** Merged image of **a** and **b**. Note that most of the P2Y₁ receptor-ir cells are also labeled with P2Y₄ immunoreactivity, such as the one indicated by a *yellow arrow*, but single P2Y₁ and P2Y₄ receptor-ir cells are also observed, such as the ones indicated by a *pink arrow* and a *blue arrow*, respectively. **d** Expression of FSH in the anterior pituitary. **e** TSH expression in the same area of **d**. **f** Merged image of **d** and **e**. Note that most of the FSH-ir cells are also labeled with TSH immunoreactivity, such as the one indicated by a *yellow arrow*, but single FSH and TSH-ir cells are also observed, such as the ones indicated by a *pink arrow* and a *blue arrow*, respectively. All scale bars=60 μ m

Table 6 Quantitative analysis of the coexistence between P2Y₁ and P2Y₄ receptors in the rat anterior pituitary (positive cell number/0.36 mm²)

	P2Y ₁ ⁺		P2Y ₁ ⁻	
	Number	%	Number	%
P2Y ₄ ⁺	42±12	93 (p1)	4±2	91 (p3)
P2Y ₄ ⁻	3±1	7 (p2)	NA	NA

P2Y₁⁺P2Y₄⁺, P2Y₁ receptor-ir cells also expressing P2Y₄ receptors; P2Y₁⁺P2Y₄⁻, P2Y₁ receptor-ir cells not expressing P2Y₄⁻; P2Y₁⁻P2Y₄⁺, P2Y₄-ir cells not expressing P2Y₁ receptors; P2Y₁⁻P2Y₄⁻, cells expressing neither P2Y₁ nor P2Y₄ receptors; NA, not assayed; p1=number of P2Y₁⁺P2Y₄⁺/total number of P2Y₁⁺ cells×100; p2=number of P2Y₁⁺P2Y₄⁻/total number of P2Y₁⁺ cells×100; p3=number of P2Y₁⁻P2Y₄⁺/total number of P2Y₄⁺ cells×100

and P2Y₄ receptors were functionally expressed in these cells. This suggestion needs to be confirmed.

The folliculo-stellate cells are glia-like cells in the anterior pituitary, which express nervous tissue-specific S100 protein [28]. The physiological functions of the folliculo-stellate cells are heterogeneous. They are involved in regulating the activity of the pituitary endocrine cells, act as immune cells such as macrophages and dendritic cells, and represent an adult stem cell population of the pituitary [29]. Previous data showed that both ATP and UTP increased the intracellular Ca²⁺ concentration of primary cultured folliculo-stellate cells of the pituitary in a concentration-dependent manner in a range between 0.1 and 10 μ M. The response was completely suppressed by thapsigargin, an inhibitor of endoplasmic reticulum

Table 7 Quantitative analysis of the coexistence between FSH and TSH in the rat anterior pituitary (positive cell number/0.36 mm²)

	FSH ⁺		FSH ⁻	
	Number	%	Number	%#
TSH ⁺	59±17	94 (p1)	5±3	92 (p3)
TSH ⁻	4±2	6 (p2)	NA	NA

FSH⁺ TSH⁺, FSH-ir cells also expressing TSH; FSH⁺ TSH⁻, FSH-ir cells not expressing TSH; FSH⁻ TSH⁺, TSH-ir cells not expressing FSH; FSH⁻ TSH⁻, cells expressing neither FSH nor TSH; NA, not assayed; p1=number of FSH⁺ TSH⁺ cells/total number of FSH⁺ cells×100; p2=the number of FSH⁺ TSH⁻ cells/total number of FSH⁺ cells×100; p3=number of FSH⁻ TSH⁺ cells/total number of TSH⁺ cells×100

Ca²⁺-ATPase, and was significantly suppressed by U-73122, an inhibitor of PLC. These results indicate that ATP increases the intracellular Ca²⁺ concentration of folliculo-stellate cells by activating PLC via P2Y₂ receptors [15]. In this study, strong immunostaining signals for P2Y₂

receptors were detected in the folliculo-stellate cells that were also stained with S100 in sections of the rat anterior pituitary. This result further confirmed the previous report that ATP/UTP, via P2Y₂ receptors released by autocrine or paracrine mechanisms, is involved in modulating the activity of folliculo-stellate cells.

In conclusion, the present study has shown that P2Y receptors are widely expressed in the anterior pituitary. P2Y₁ and P2Y₄ receptors are found to be expressed in the majority of gonadotrophs and thyrotrophs, P2Y₂ receptors are expressed in a small subpopulation of lactotrophs and almost all of the folliculo-stellate cells that are stained with S100 protein. The identity of a small subpopulation of P2Y₁₃ receptor-ir cells in the rat anterior pituitary still needs to be confirmed. The present results show that P2Y₁ and P2Y₄ receptors are co-expressed in a subpopulation of gonadotrophs and thyrotrophs. Purines and pyrimidines may act on gonadotrophs and thyrotrophs at the same time via P2Y₁ and P2Y₄ receptors. This result further confirmed the previous report that ATP/UTP, via P2Y₂ receptors

Fig. 6 Results of Western blotting (WB) and control experiments. **a** WB results. The color ladder is the molecular marker of protein; lanes 1, 3, 5, 7, 8, 9 are the results of P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂, and P2Y₁₃ WB, respectively. Note that immunoreactive bands were detected at different molecular weight levels: one band for P2Y₁ receptors at about 48 kDa (lane 1), two bands for P2Y₂ receptors at about 36, 90 kDa (lane 3), one band for P2Y₄ at about 49 kDa (lane 5), no clear band for P2Y₆ or P2Y₁₂ receptors (lane 7, lane 8), one band for P2Y₁₃ at about 37 kDa (lane 9); lanes 2, 4, 6 and 10 are the results of P2Y₁, P2Y₂, P2Y₄, and P2Y₁₃ peptide pre-absorption experiments. Note that pre-adsorption of the antisera with the peptide antigens resulted in the absence of bands (lanes 2, 4, 6, 10). Control experiments were carried out with the P2Y antibodies pre-absorbed with their respective peptides. No staining was observed in those preparations incubated with antisera solutions pre-absorbed with their respective peptides, as shown for P2Y₂ and P2Y₄ receptor antisera (b, c). Scale bars=60 μm

released by an autocrine or paracrine mechanism, is involved in modulating the activity of folliculo-stellate cells.

References

- Burnstock G (2007) Purine and pyrimidine receptors. *Cell Mol Life Sci* 64:1471–1483
- Tomic M, Jobin RM, Vergara LA, Stojilkovic SS (1996) Expression of purinergic receptor channels and their role in calcium signaling and hormone release in pituitary gonadotrophs. Integration of P2 channels in plasma membrane- and endoplasmic reticulum-derived calcium oscillations. *J Biol Chem* 271:21200–21208
- Nunez L, Villalobos C, Frawley LS (1997) Extracellular ATP as an autocrine/paracrine regulator of prolactin release. *Am J Physiol* 272:E1117–E1123
- Koshimizu TA, Tomic M, Wong AO, Zivadinovic D, Stojilkovic SS (2000) Characterization of purinergic receptors and receptor-channels expressed in anterior pituitary cells. *Endocrinology* 141:4091–4099
- Villalobos C, Alonso-Torre SR, Nunez L, Garcia-Sancho J (1997) Functional ATP receptors in rat anterior pituitary cells. *Am J Physiol* 273:C1963–C1971
- Chung HS, Park KS, Cha SK, Kong ID, Lee JW (2000) ATP-induced $[Ca^{2+}]^{(i)}$ changes and depolarization in GH3 cells. *Br J Pharmacol* 130:1843–1852
- Stojilkovic SS, Koshimizu T (2001) Signaling by extracellular nucleotides in anterior pituitary cells. *Trends Endocrinol Metab* 12:218–225
- Zemkova H, Balik A, Jiang Y, Kretschmannova K, Stojilkovic SS (2006) Roles of purinergic P2X receptors as pacemaking channels and modulators of calcium-mobilizing pathway in pituitary gonadotrophs. *Mol Endocrinol* 20:1423–1436
- Zemkova H, Kucka M, Li S, Gonzalez-Iglesias AE, Tomic M, Stojilkovic SS (2010) Characterization of purinergic P2X4 receptor channels expressed in anterior pituitary cells. *Am J Physiol Endocrinol Metab* 298:E644–E651
- van der Merwe PA, Wakefield IK, Fine J, Millar RP, Davidson JS (1989) Extracellular adenosine triphosphate activates phospholipase C and mobilizes intracellular calcium in primary cultures of sheep anterior pituitary cells. *FEBS Lett* 243:333–336
- Davidson JS, Wakefield IK, Sohnius U, van der Merwe PA, Millar RP (1990) A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology* 126:80–87
- Chen ZP, Kratzmeier M, Poch A, Xu S, McArdle CA, Levy A, Mukhopadhyay AK, Lightman SL (1996) Effects of extracellular nucleotides in the pituitary: adenosine triphosphate receptor-mediated intracellular responses in gonadotrope-derived alpha T3-1 cells. *Endocrinology* 137:248–256
- He ML, Gonzalez-Iglesias AE, Tomic M, Stojilkovic SS (2005) Release and extracellular metabolism of ATP by ecto-nucleotidase eNTPDase 1–3 in hypothalamic and pituitary cells. *Purinergic Signal* 1:135–144
- He ML, Gonzalez-Iglesias AE, Stojilkovic SS (2003) Role of nucleotide P2 receptors in calcium signaling and prolactin release in pituitary lactotrophs. *J Biol Chem* 278:46270–46277
- Uchiyama M, Nakajima Y, Sakuma Y, Kato M (2001) Purinergic regulation of intracellular Ca^{2+} concentration of rat pituitary folliculo-stellate cells in primary culture. *J Neuroendocrinol* 13:378–385
- Chen ZP, Krull N, Xu S, Levy A, Lightman SL (1996) Molecular cloning and functional characterization of a rat pituitary G protein-coupled adenosine triphosphate (ATP) receptor. *Endocrinology* 137:1833–1840
- Carew MA, Wu ML, Law GJ, Tseng YZ, Mason WT (1994) Extracellular ATP activates calcium entry and mobilization via P2U-purinoreceptors in rat lactotrophs. *Cell Calcium* 16:227–235
- Teramoto N, Szekely L, Pokrovskaja K, Hu LF, Yoshino T, Akagi T, Klein G (1998) Simultaneous detection of two independent antigens by double staining with two mouse monoclonal antibodies. *J Virol Meth* 73:89–97
- Xiang Z, Burnstock G (2005) Distribution of P2Y₂ receptors in the guinea pig enteric nervous system and its coexistence with P2X₂ and P2X₃ receptors, neuropeptide Y, nitric oxide synthase and calretinin. *Histochem Cell Biol* 124:379–390
- Xiang Z, He C, Burnstock G (2006) P2X₅ receptors are expressed on neurons containing arginine vasopressin and nitric oxide synthase in the rat hypothalamus. *Brain Res* 1099:56–63
- Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology. Update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 58:281–341
- Troadec JD, Thirion S (2002) Multifaceted purinergic regulation of stimulus-secretion coupling in the neurohypophysis. *Neuroendocrinol Lett* 23:273–280
- Chen ZP, Kratzmeier M, Levy A, McArdle CA, Poch A, Day A, Mukhopadhyay AK, Lightman SL (1995) Evidence for a role of pituitary ATP receptors in the regulation of pituitary function. *Proc Natl Acad Sci USA* 92:5219–5223
- Burnstock G (2004) Cotransmission. *Curr Opin Pharmacol* 4:47–52
- Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* 50:413–492
- Chen ZP, Levy A, McArdle CA, Lightman SL (1994) Pituitary ATP receptors: characterization and functional localization to gonadotrophs. *Endocrinology* 135:1280–1283
- Hirano N, Shiino M (1993) Co-existence of gonadotrophins (FSH, LH) and thyrotrophin (TSH) in single anterior pituitary cells of the musk shrew, *Suncus murinus*. *Cell Tissue Res* 272:315–320
- Nakajima T, Yamaguchi H, Takahashi K (1980) S100 protein in folliculostellate cells of the rat pituitary anterior lobe. *Brain Res* 191:523–531
- Allaerts W, Vankelecom H (2005) History and perspectives of pituitary folliculo-stellate cell research. *Eur J Endocrinol* 153 (1):1–12