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P2Y purinoceptors in gastric gland plasma membranes

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Abstract

This autoradiographic study of sections of the rabbit stomach fundus labelled with [^{35}S]dATPαS, a radioligand for P2Y purinoceptors, has demonstrated a discrete pattern of distribution of the binding sites, i.e., the specific binding was only over the mucosa, but not over the muscular layer. Radioligand binding assays carried out on gastric gland plasma membranes showed that the binding process was saturable and a high density of a homogeneous population of binding sites was observed. These binding sites presented high affinity with a value of $K_d = 4.1 \pm 0.8$ nM and the maximum density of the binding sites was 16.8 ± 1.6 pmol/mg protein. The displacement by purinoceptor ligands showed the following order of potency: ATP = 2-methylthio ATP ≫ α,β-methylene ATP ≫ adenosine. Neither UTP nor pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) were able to displace the binding. The data support the presence of P2Y purinceptors in rabbit gastric glands.

Keywords: P_{2Y} purinoceptor; ATP; Gastric mucosa; Plasma membrane

1. Introduction

Knowledge of the physiological functions of extracellular purines has increased substantially during the last decade. ATP, adenosine and some other purine derivatives play a role in neurotransmission, vascular secretion and immunosystems (Gordon, 1986; Olsson and Pearson, 1990; Burnstock, 1993, 1995). The first classification of the receptors that mediate the effects of extracellular purines was proposed by Burnstock (1978). These receptors were divided into two major types, terming P_1 and P_2 purinoceptors, preferentially activated by adenosine and ATP, respectively. Further experiments have been carried out to support and extend the P_1/P_2 classification (Burnstock, 1991; Abbracchio et al., 1993; Fredholm et al., 1994). In a recent paper (Abbracchio and Burnstock, 1994) a flexible framework for the classification of P_2 purinoceptors was proposed, that divides the actions of adenine nucleotides into two broad purinoceptor families, P2X and P2Y. The main difference between these purinoceptor families can be summarised on the basis of their transduction mechanisms: the P2X purinoceptor subtype involves an intrinsic ion channel permeable to Na^+, K^+ and Ca^{2+}, whereas the P2Y purinoceptor subtype is a G-protein-coupled receptor that modulates membrane phosphoinositide metabolism, diacyl-glycerol (DAG) generation, as well as modulation of cAMP generation and arachidonic acid mobilization. However, selective agonists and antagonists are still not clearly defined for these receptors. In general, it seems that the ATPγS and α,β-methylene ATP (α,β-MeATP) and similar ATP derivatives are good agonists for P2X purinoceptors, while N^6-methyl and 2-thioether ATP derivatives are good agonists for P2Y purinoceptors (Bo et al., 1994; Williams, 1995).

P_2 purinoceptors (in most cases the P2Y subtype, mediating relaxation) have so far been identified in stomach (Hoyle and Burnstock, 1991; Burnstock et al., 1994). However, little information is available about the effects of ATP on gastric acid secretion. In early works carried out using amphibian gastric mucosa, ATP was found to inhibit histamine-stimulated acid secretion (Kidder, 1971; Sanders et al., 1976). Studies on rabbit gastric glands show that ATP causes inhibition of the histamine-stimulated H^+ secretion showing the rank order of potency ATP > ADP ≫ β,γ-methylene ATP (β,γ-MeATP) (Gil-Rodrigo et al., 1990) and such an effect can be significantly reduced by the prostaglandin synthesis inhibitor, indomethacin (Gil-Rodrigo et al., 1993).

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The aim of this study was to investigate the presence of P2Y purinoceptors in the rabbit gastric mucosa which may be involved in acid secretion. We carried out a receptor binding assay with [^{35}S]2′-deoxyATPαS ([^{35}S]dATPαS) on plasma membranes of isolated gastric glands. In addition, autoradiographic localisation of [^{35}S]dATPαS binding sites was conducted in the sections of rabbit stomach fundus.

2. Materials and methods

2.1. Radioligand binding assay

New Zealand rabbits weighing 2.5–4.0 kg were killed by an overdose of sodium pentobarbital in an injection through the marginal ear vein, followed by exsanguination. The stomach was then perfused with phosphate-buffered saline and removed. The gastric glands were isolated as described by Berglindh and Obrink (1976).

A crude fraction of gastric gland plasma membranes was obtained as described by Maeda et al. (1983). The gastric gland homogenate was layered over 10 ml of 41% sucrose. After 1 h of centrifugation at 95 000 × g, a white interfacial band of membranes was collected. These membranes were pelleted by centrifugation at 95 000 × g for 20 min, washed twice and kept at −80°C. Protein concentration was measured using the method of Lowry et al. (1951) and bovine serum albumin was used as the standard.

For the saturation experiments [^{35}S]dATPαS in a concentration range 1–100 nM was used. To determine the non-specific binding, 100 μM of 2-methylthio ATP (2-MeSATP) was added in parallel with the tubes for total binding. The incubation was carried out in 50 mM Tris-HCl buffer containing 1 mM EGTA, 1 mM benzamidine hydrochloride, 0.1 mM phenylmethylsulfonyl fluoride, 0.01% bacitracin, and 0.002% soybean trypsin inhibitors (Buffer A, pH 7.4) at room temperature for 1 h. The reaction was terminated by quick filtration of the mixture through GF/B filters presoaked in 20 mM disodium pyrophosphate solution. The filters were washed twice with 1 ml of ice-cold Tris-HCl buffer. The radioactivities in the filters were counted in a Beckman SC6000I scintillation counter (Beckman, Fullerton, CA, USA). Each experiment was carried out in duplicate.

2.2. Autoradiography

New Zealand rabbits were killed as described above. Small pieces of the stomach fundus wall were removed, placed in Krebs solution and trimmed. Sections of 14 μm thickness were cut and thaw-mounted onto acid-washed, gelatin-coated slides.

Slide-mounted sections were preincubated in Buffer A at 30°C for 10 min to remove endogenous ligands and embedding matrix. They were then transferred to Buffer A

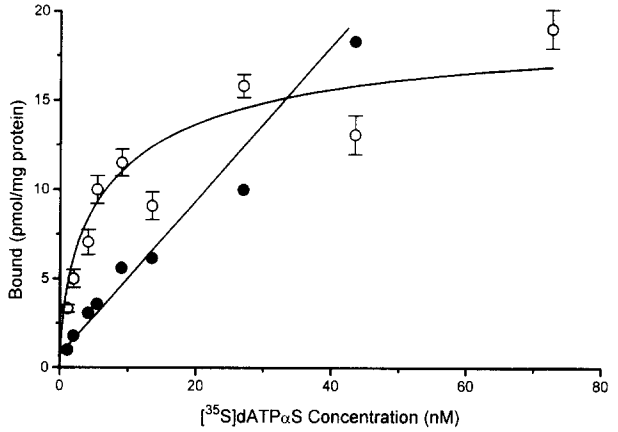

Fig. 1. Saturation curve of [^{35}S]dATPαS binding to rabbit gastric gland plasma membranes. Non-specific binding was detemined in the presence of 100 μM of 2-MeSATP. Incubation was carried out at room temperature for 1 h. Each point represents the mean of four duplicated experiments. Error bars show S.E.M.

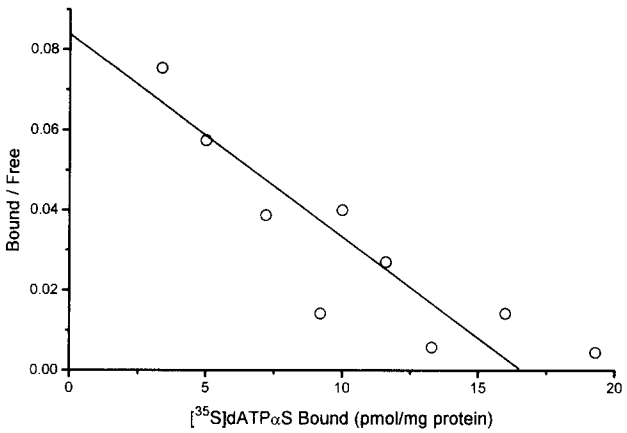

Fig. 2. Scatchard plot fitted by the computer programme RADLIG. One binding site model was chosen based on the results of the F-test of weighted residual sum squares.

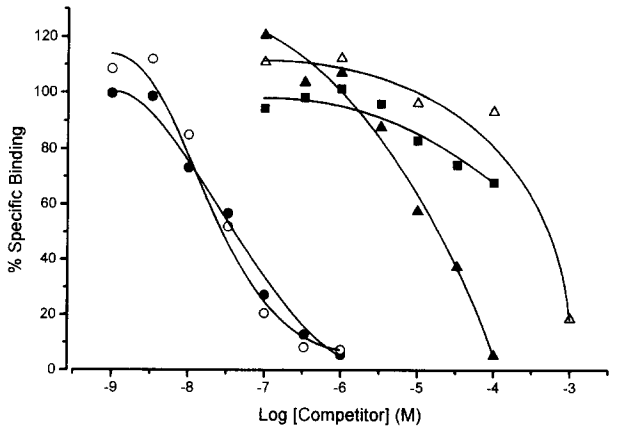

Fig. 3. Displacement by purinoceptor ligands. (●) 2-MeSATP, (○) ATP, (▲) α,β-meATP, (△) adenosine and UTP (■). 100% corresponds with the specific binding of 10 nM of [^{35}S]dATPαS. The standard errors were all less than 15% of the means.

containing 10 nM [^{35}S]dATPαS and incubated at room temperature for 1 h. Non-specific binding was determined in the presence of 100 mM 2-MeSATP. At the end of the incubation, the slides were washed in ice-cold Tris-HCl buffer for 2 × 2 min and in ice-cold double-distilled water for 1 min. Sections were dried under cold airflow and

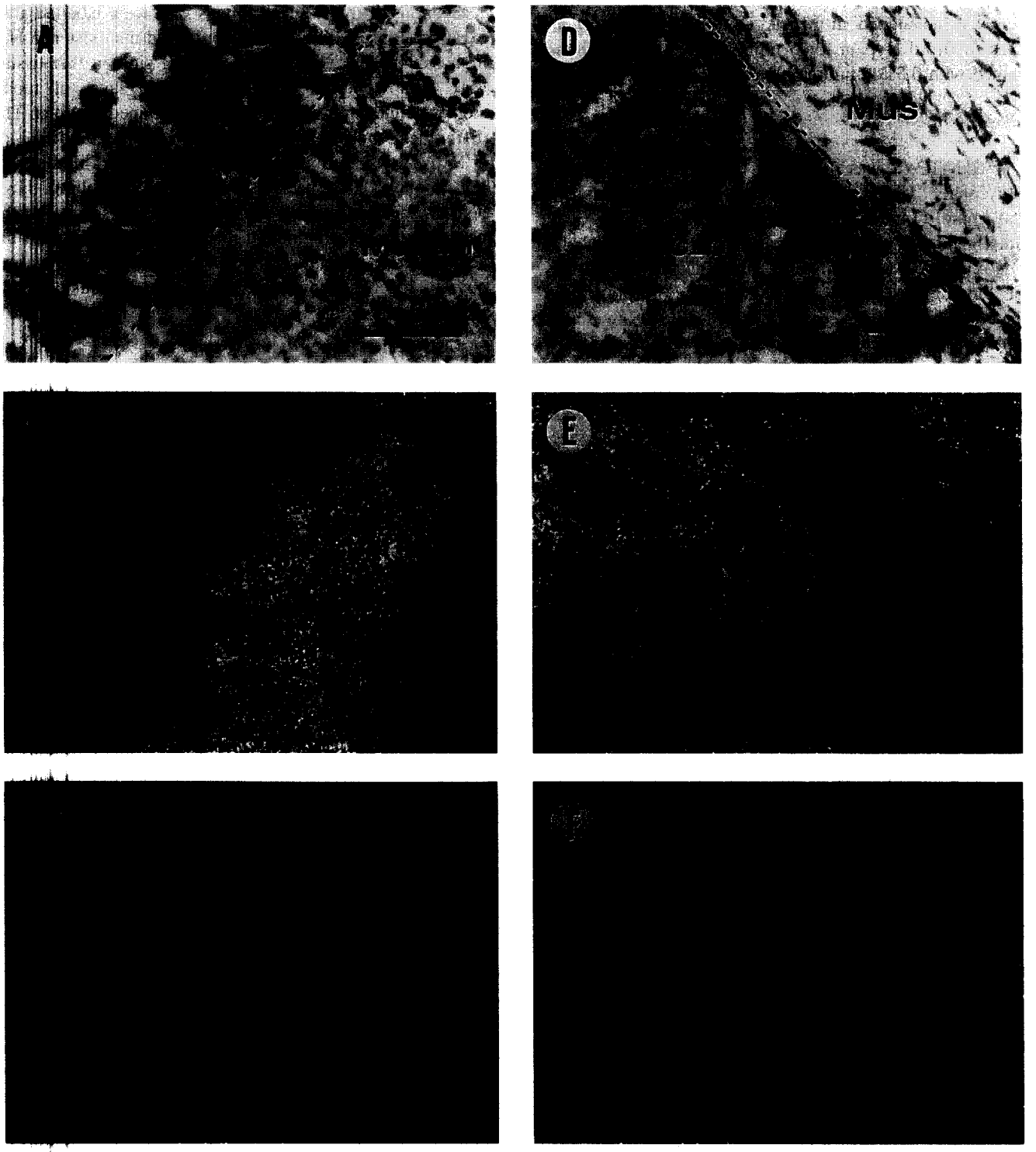

Fig. 4. Autoradiographs of the rabbit stomach fundus. (A,D) Bright-field views of two sections (14 μm thick, stained with 0.5% toluidine blue). (B,E) Dark-fields views of (A) and (D), which show the overall distribution of [^{35}S]dATPαS binding sites in the sections. (C,F) Dark-fields views of the adjacent sections of (A) and (D) showing the non-specific binding sites of [^{35}S]dATPαS in these sections. Non-specific binding was determined in the presence of 100 μM 2-MeSATP. Incubation was carried out at room temperature for 1 h. Mus, muscularis; S, surface cells of mucusa; Muc-n, neck region of mucusa; Muc-b, base region of mucusa. Scale bar = 100 μm.

stored in a desiccator at 4°C. The time and temperature for the incubation and the wash had been optimised in preliminary experiments.

An emulsion-coated coverslip (Ilford K5, diluted 1 : 1 with distilled water) was attached to one end of each slide. The assemblies were exposed for 24 h at 4°C. The emulsion was developed in Kodak D-19 (20°C, 3 min) and fixed in Ilford Hypam for 5 min. The sections were then stained with 0.5% toluidine blue.

2.3. Materials

[^{35}S]dATPαS was purchased from Du Pont NEN UK (Stevenage, UK), with a specific activity of 54.3 Tbq/mmol (1468 Ci/mmol). The specific activity of the radioligand was reduceded by 100 times with non-labelled 2-deoxyATPαS (Amersham International, Amersham, UK) for binding assay and autoradiography. Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) was a gift from Prof. Lambrecht, University of Frankfurt, Germany. 2-MeSATP was from Research Biochemicals International (Natick, USA). Other compounds were purchased from Sigma Chemicals (Poole, UK).

2.4. Statistical analyses

The binding data were analysed with a computer program RADLIG (Biosoft, Cambridge, UK). The choice of binding model was based on the results of an *F*-test on the weighted residual sums of squares and standard errors (Munson and Rodbard, 1980).

3. Results

3.1. Radioligand binding assay

The binding of [^{35}S]dATPαS to the rabbit gastric plasma membranes was saturable (Fig. 1). Curve-fitting showed only one affinity binding site existed in the preparations (Fig. 2). The maximum density of binding sites was 16.8 ± 1.6 pmol/mg protein, and the apparent dissociation constant (K_d) was 4.1 ± 0.8 nM ($n = 4$). Hill coefficients were around 1, indicating the absence of co-operation.

3.2. Competition studies

The binding of the radioligand (10 nM) to plasma membranes of three different sets of the gastric glands was studied in the presence of increasing concentrations of several ATP analogues: 2-MeSATP, α,β-MeATP, PPADS, ATP, adenosine and UTP. ATP and 2-MeSATP were the most potent ligands tested to displace the binding of [^{35}S]dATPαS, with IC_{50} values of 25.0 ± 0.2 nM and 28.0 ± 0.4 nM, respectively (Fig. 3). α,β-MeATP and adenosine were much less potent than ATP and 2-MeSATP, with IC_{50} values of 38.0 ± 8.4 μM and 220 ± 56 μM, respectively (Fig. 3). Neither UTP nor PPADS displaced the radioligand more than 30% at the highest concentration used (100 μM).

3.3. Autoradiography

Sections of the stomach fundus wall were labelled after exposure for just 24 h, which confirmed the existence of a high density of [^{35}S]dATPαS binding sites in that tissue. Fig. 4 shows the mucosa and muscle layer of rabbit stomach fundus on the toluidine blue-stained sections. The difference between panel B,E (total binding of [^{35}S]dATPαS) and panel C,F (non-specific binding) in Fig. 4 shows the specific [^{35}S]dATPαS labelling. High density specific binding sites were mainly seen over the neck and the base regions of mucosa which are rich in the acid-secreting parietal cells (Fig. 4B,E), while there was no obvious specific binding over the surface mucous cells and the muscularis (Fig. 4B,E).

4. Discussion

Following the review by Abbracchio and Burnstock (1994), several P2Y purinoceptor family subtypes involving in G-protein activation have been described in a remarkable variety of tissues and cell types. Evidence has been presented for the involvement of P2Y purinoceptors in the regulation of surfactant phospholipid secretion in alveolar type II cells and cystic fibrosis (Parr et al., 1994; Rice et al., 1995), as well as on the endocrine function of thyroid and parathyroid cells (Sato et al., 1992; Gibb et al., 1994); pituitary cells (Carew et al., 1994); chromaffin cells (Castro et al., 1995) and pancreatic β cells (Hillaire-Buys et al., 1993). In hepatocytes (Keppens et al., 1992) and in the terminal inner medullary collecting duct of kidney (Ecelbarger et al., 1994) the existence of this purinoceptor subtype has also been proposed, where ATP acts directly on renal mesangial cells and on epitheliod MDCK-cells (Paulmichl et al., 1991; Parr et al., 1994). Two P2Y purinoceptor family subtypes, namely $P2Y_1$ and $P2Y_2$ (formerly P_{2u}) have been identified in endothelial cells (Burnstock, 1990; Boeynaems and Pearson, 1990; Vigne et al., 1994; Parr et al., 1994; Sipma et al., 1995).

The role of ATP and its analogues on the gastro-intestinal epithelial cells has already been reported. It has been suggested that a P_2 purinoceptor subtype is linked to adenylate cyclase and electrolyte transport in enterocytes (Korman et al., 1982), stimulating mucus secretion with an increase in the intracellular Ca^{2+} concentration in rabbit gastric mucus cells (Ota et al., 1994) and further that they are involved in modulation of gastric acid secretion stimulated by histamine in rabbit gastric glands and parietal cells (Gil-Rodrigo et al., 1990, 1993).

The autoradiographs presented in this study confirm the existence of high density binding sites of [^{35}S]dATPαS on the fundus mucosa of the rabbit stomach. Binding studies

on gastric gland plasma membranes showed that receptors are abundant too. A homogeneous population of specific binding sites has been detected, with a B_{max} value of 16.8 pmol/mg protein. The K_d value of 4.1 nM shows high affinity of the radioligand for the P2Y purinoceptor.

Further information about these specific binding sites can be inferred from the concentration-dependent competition of [^{35}S]dATPαS binding by ATP and other purinoceptor ligands. The following displacement potency order has been observed: ATP = 2-MeSATP ≫ α,β-MeATP ≫ adenosine. Neither UTP nor PPADS were able to displace the radioligand binding by more than 30% at 0.1 mM (10^4-fold of radioligand concentration).

This rank potency order agrees in part with that reported by Gil-Rodrigo et al. (1990 and personal communication) where the histamine-stimulated acid secretion in isolated rabbit gastric glands was inhibited by ATP and analogues with the potency order of ATP > ADP ≥ 2-MeSATP ≫ β,γ-MeATP and this effect is abolished by indomethacin (Gil-Rodrigo et al., 1993).

Neither α,β-MeATP examined at the plasma membrane level nor β,γ-MeATP on gastric gland acid secretion were effective analogues, although potent agonists for the P2X purinoceptor family. This is consistent with the report by Ota et al. (1994) for rabbit gastric mucous cells. Thus it seems likely that the specific binding sites localized on gastric plasma membranes are of the P2Y purinoceptor family.

The inefficient competition of the specific binding sites by UTP could indicate that the $P2Y_2$ purinoceptor, the most common purinoceptor described in different cell types involved in secretion processes, could be ruled out as the binding site described in this study.

In spite of the limited information available on binding studies for P2Y radioligands, it appears that the low K_d value obtained (4.1 nM) differs from values (about 60–100-fold higher) calculated for P_2 purinoceptors in rat hepatocytes (Keppens and De Wulf, 1986) and hamster tracheal epithelial cells (Kim et al., 1994), using ATPα^{35}S and ATPγ^{35}S as radioligands. However it is similar to the K_d value obtained for $P2Y_1$ purinoceptor in chick brain plasma membranes (K_d = 10 nM) and the same receptor expressed in COS-7 cells (K_d = 7 nM). Also the agonist competition order described in the present study is quite similar to that reported for $P2Y_1$ (Webb et al., 1994) with the IC_{50} values of 81 nM, 60 nM, > 10 μM, > 10 μM for ATP, 2-MeSATP, α,β-MeATP and UTP respectively, in chick brain membranes and 99 nM, 142 nM, > 10 μM, > 10 μM in COS-7 cells.

Taking into account this evidence and the probable involvement of G-protein in antagonizing the histamine-stimulated acid secretion, which is inhibited by indomethacin in gastric glands, it is suggested that a P2Y purinoceptor subtype is present in the rabbit gastric gland.

Further investigation will be carried out to study the pathway involved in the activation of this purinoceptor at the plasma membrane level and on the process of acid secretion, and establish the P2Y purinoceptor subtype present in the rabbit gastric gland.

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