

Signaling by extracellular nucleotides in anterior pituitary cells

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Pituitary cells secrete ATP, which acts as an autocrine and/or paracrine extracellular messenger on two families of purinergic receptors: G-protein-coupled P2Y receptors (P2YRs) and ion-conducting P2X receptors (P2XRs). Lactotrophs and GH₃-immortalized cells express the P2Y₂R subtype. Several P2XR subtypes are expressed in pituitary cells. Gonadotrophs and somatotrophs express P2X_{2a}R and P2X_{2b}R, which occur as heteromeric channels. Lactotrophs and GH₃ cells express one or more ion-conducting subtypes from among P2X₃R, P2X₄R and P2X₇R in homomeric form. Thyrotrophs and corticotrophs also express P2XRs, but their identification requires further study. Pituitary cells express purinergic P1 receptors, which are activated by adenosine. The A₁R subtype of these receptors is expressed in melanotrophs and GH₃ cells. In this review, we briefly discuss the expression and coupling of A₁R and P2Y₂R, and focus on the expression and Ca²⁺ signaling of P2XRs.

Purines (ATP, ADP and adenosine) and pyrimidines (UTP and UDP) act as extracellular messengers via plasma membrane receptors termed purinergic receptors (PRs). There are two main groups of purinergic receptor: adenosine-activated P1 receptors (P1Rs) and ATP-, ADP-, UTP- and/or UDP-activated P2 receptors (P2Rs). This division was originally introduced by Burnstock¹, and the terms P1Rs and 'adenosine receptors' (ARs) are synonymous. Molecular, physiological and pharmacological evidence suggests the existence of four mammalian AR subtypes (denoted A₁R, A_{2A}R, A_{2B}R and A₃R), which all signal through G-protein-dependent pathways. A₁R is negatively coupled to the adenylyl cyclase signaling pathway through pertussis toxin-sensitive G_{i/o}. In some tissues, A₁R stimulates phospholipase C (PLC) and D (PLD) signaling pathways, and modulates plasma membrane channel activity. By contrast, A₂R signals through the cholera toxin-sensitive G_s-adenylyl cyclase signaling pathway, but also activates G_{q/11}-dependent PLC and modulates the activity of voltage-gated Ca²⁺ channels and/or ATP-regulated K⁺ channels. The signal transduction mechanism of A₃R has not been completely characterized².

P2 receptors belong to one of two families: G-protein-coupled receptors [P2Y receptors (P2YRs)], originally identified by Dubyak³, and ligand-gated ion channels [P2X receptors (P2XRs)], identified by Benham and Tsien⁴. So far, five mammalian P2YRs have been cloned and are denoted P2Y₁R, P2Y₂R, P2Y₄R, P2Y₆R and P2Y₁₁R. Three of them, P2Y₁R, P2Y₂R and P2Y₆R, signal through G_{q/11} pathways, leading to activation of PLC and the generation of inositol (1,4,5)-trisphosphate

[Ins(1,4,5)P₃] and diacylglycerol. In excitable cells, Ins(1,4,5)P₃-induced Ca²⁺ mobilization is frequently accompanied by cell-specific modulation of voltage-gated Ca²⁺ influx through Ca²⁺-activated K⁺ channels and/or voltage-gated Ca²⁺ channels. Stimulation of mitogen-activated protein (MAP) kinase and PLD signaling pathways, both secondary to the activation of protein kinase C (PKC), has been reported for P2Y₂R. Cross-coupling of P2Y₁R to the G_{i/o} signaling pathway has also been observed. Further studies are required to clarify the coupling of other P2YRs (Ref. 2).

With the use of molecular cloning techniques, seven P2XR subtypes have been identified to date, denoted P2X₁R to P2X₇R, and several spliced forms have been observed⁵⁻¹¹. P2XR subtypes differ with respect to their ligand-selectivity profiles, antagonist sensitivity and cation selectivity. Their activation leads to an increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i), with Ca²⁺ influx occurring through the pores of these channels and through voltage-gated Ca²⁺ channels, following the initial depolarization of cells by P2XR-generated currents. They can form ion-permeable pores through homo- and heteropolymerization^{12,13}. Each subunit is proposed to have two transmembrane helices (M1 and M2) connected by a large extracellular loop, with both N- and C-termini located in the cytoplasm. From the N-terminus to the second transmembrane domain, the cloned subunits exhibit a relatively high level of amino acid sequence homology. By contrast, the C-termini vary in length and show no apparent sequence homology, except for the region nearest to the second transmembrane domain¹⁴.

The physiological role of purines and pyrimidines as extracellular messengers was established by the finding that several tissues release them in a regulated manner². The roles of ATP as a neurotransmitter or co-transmitter, and adenosine as an important neuromodulator are well established in peripheral and central nervous systems. In addition, cardiac tissue also releases adenosine, whereas ATP is secreted by skeletal muscle, adrenal chromaffin cells, mast cells, blood cells, fibroblasts and endothelial cells. Several reports have also suggested that pyrimidines are secreted by endothelial, epithelial and astrocytoma cells². 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

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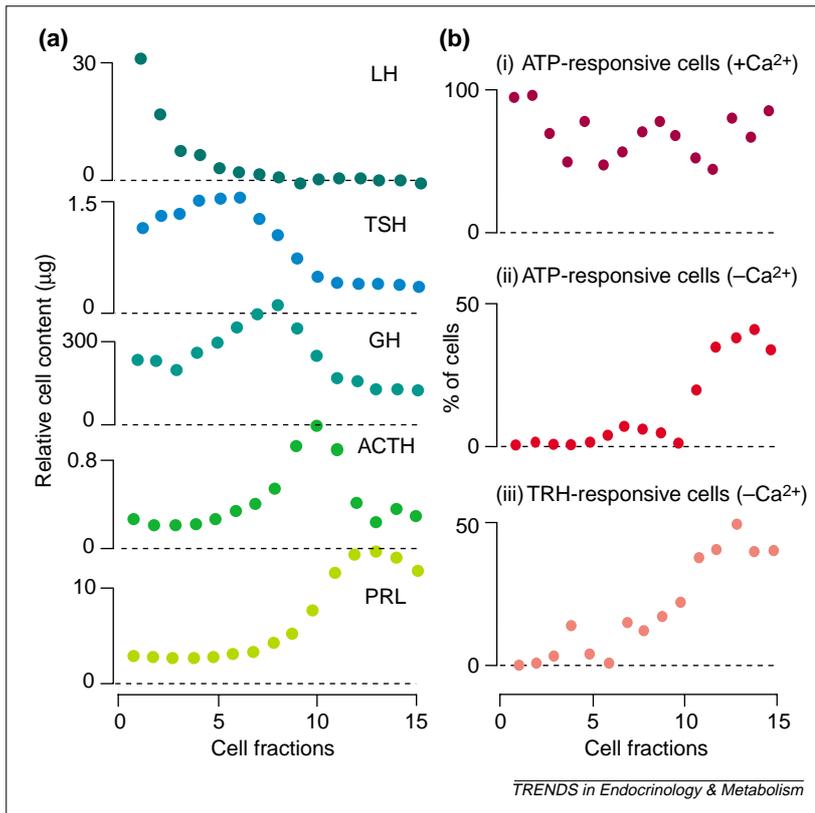


Fig. 1. Purification of secretory anterior pituitary cells and the distribution of purinergic receptors and receptor-channels in purified fractions. (a) Hormonal contents of purified pituitary cells. Dispersed cells were separated by FicolTM gradients into 15 fractions. Cells were washed, counted and 10^5 cells from each fraction were dialyzed immediately after separation to measure their hormonal contents. (b) Ca^{2+} response in purified pituitary cells. Percentage of cells responding to ATP with a rise in intracellular $[Ca^{2+}]_i$ when bathed in (i) a Ca^{2+} -containing medium and when bathed in (ii) a Ca^{2+} -deficient medium and (iii) percentage of cells responding to TRH with a rise in intracellular $[Ca^{2+}]_i$ when bathed in a Ca^{2+} -deficient medium. Abbreviations: ACTH, adrenocorticotrophic hormone; GH, growth hormone; LH, luteinizing hormone; PRL, prolactin; TRH, thyrotropin-releasing hormone; TSH, thyrotropin.

activation of PRs. These enzymes degrade ATP to ADP, AMP and adenosine^{15,16}.

Adenosine receptors

Immortalized rat pituitary GH₃ cells and frog melanotrophs express P1Rs. The structure of these receptors has not been identified, but pharmacological, electrophysiological and secretory data indicate the expression of the A₁ subtype of these receptors, which is negatively coupled to the adenylyl cyclase signaling pathway through pertussis toxin-sensitive G proteins¹⁷. In immortalized pituitary cells, its activation causes abolition of spontaneous electrical activity and Ca^{2+} signaling^{18,19}, as well as the inhibition of prolactin (PRL) and growth hormone (GH) secretion^{17,20}. The abolition of spontaneous electrical activity by adenosine occurs indirectly, by activation of voltage-gated K⁺ channels, and/or directly, by inhibition of voltage-gated Ca^{2+} channels^{18,21}. Two reports have also indicated the operation of A₁R in pituitary lactotrophs^{22,23}, but further studies are required to clarify its structure, coupling and effects (stimulatory or inhibitory) on PRL secretion.

Distribution of P2YRs and P2XRs within secretory anterior pituitary cells

Figure 1 illustrates the relative purification of rat secretory anterior pituitary cells by FicolTM gradient centrifugation (Fig. 1a) and the expression pattern of P2YRs and P2XRs in such purified cells (Fig. 1b). The FicolTM gradient procedure for the enrichment of cells generates 15 fractions²⁴, and the measurement of the hormonal content of these fractions revealed that the first three fractions predominantly contain luteinizing hormone (LH), fractions 3–5 contain thyrotropin (TSH), fractions 4–10 contain GH, fractions 8–11 contain adrenocorticotrophic hormone (ACTH) and fractions 11–15 contain PRL. To characterize the distribution of Ca^{2+} -mobilizing P2YRs and ion influx-dependent P2XRs within these fractions, cells were stimulated with ATP in Ca^{2+} -containing and Ca^{2+} -deficient medium. In Ca^{2+} -containing medium, an ATP-induced $[Ca^{2+}]_i$ response was seen in 50–90% of the cells in different fractions (Fig. 1bi). When bathed in Ca^{2+} -deficient medium, however, the rise in $[Ca^{2+}]_i$ was observed in ~40% of cells from fractions 11–15 (Fig. 1bii), and cells from these fractions also responded to thyrotropin-releasing hormone when bathed in a Ca^{2+} -deficient medium (TRH; Fig. 1biii). These results indicate that all five main secretory cell types expressed P2XRs capable of facilitating Ca^{2+} influx, whereas Ca^{2+} -mobilizing P2YRs are predominantly expressed in lactotrophs. A small percentage of unidentified cells from somatotroph and corticotroph fractions also express P2YRs.

Characterization of pituitary P2YRs

The amino acid sequence and the signal transduction pathways of pituitary P2YRs were characterized by Lightman's group²⁵. These receptors are Ca^{2+} -mobilizing P2Y₂R subtypes coupled to the PLC signaling pathway by G_{q/11}. Similar to other cell types expressing P2Y₂R (Refs 26,27), their activation in pituitary cells leads to the stimulation of Ins(1,4,5)*P*₃ and diacylglycerol production, Ca^{2+} release from intracellular stores and the translocation of PKC (Refs 25,28–31). In accordance with results shown in Fig. 1, RT-PCR using P2Y₂R primers indicated the presence of a PCR fragment with the expected size of P2Y₂R in enriched lactotrophs (Fig. 2a, lane 3) and immortalized GH₃ cells (lane 4), but not in enriched somatotrophs and gonadotrophs²⁴.

The expression of other P2YRs in pituitary tissue has not been investigated. However, P2YRs expressed in lactotrophs respond to ATP, UTP, a specific agonist for P2Y₂R and P2Y₄R, and adenosine-5'-O-(3-thiotriphosphate) (ATP- γ -S) when bathed in a Ca^{2+} -deficient medium. The relative potency of ATP agonists for these receptors is ATP = UTP > ATP- γ -S. Furthermore, 3'-O-(4-benzoyl)benzoyl-ATP (BzATP), a relatively specific agonist for P2X₇R (Ref. 2), is unable to initiate Ca^{2+} signaling in lactotrophs. Several other ATP analogs, including α,β -methylene

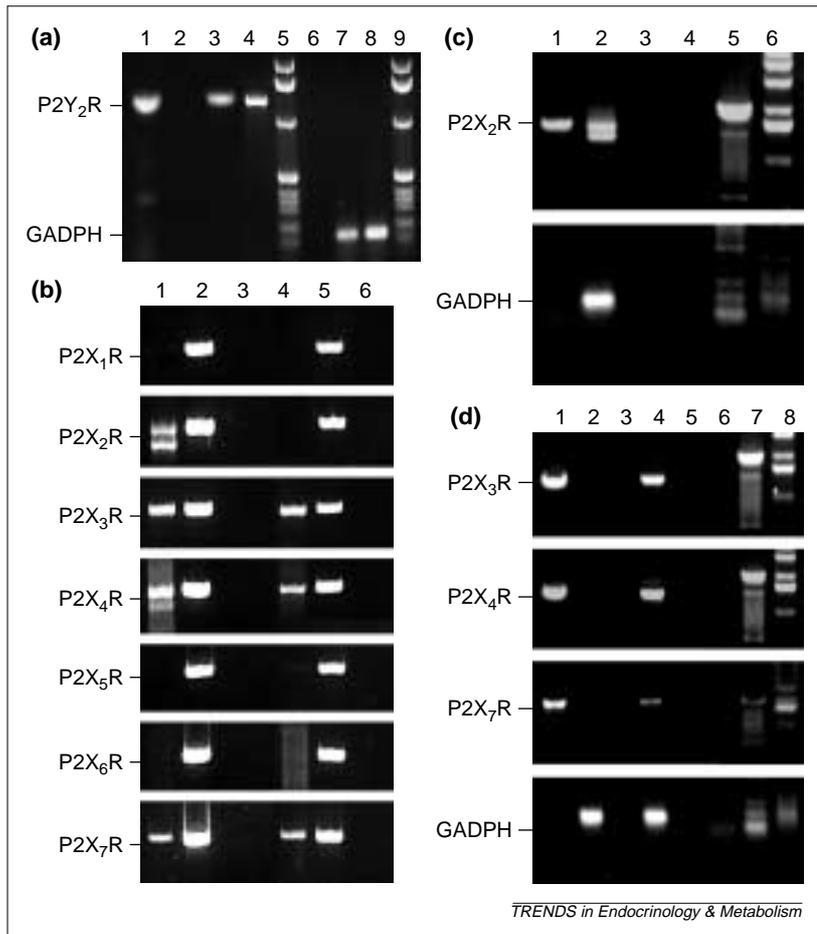


Fig. 2. Expression of G-protein-coupled purinergic receptors (P2YRs) and ion-conducting purinergic receptors (P2XRs) in anterior pituitary cells. (a) RT-PCR analysis of mRNA encoding P2Y₂R in enriched lactotrophs (lane 3) and GH₃ cells (lane 4). Total RNA isolated from the rat brain was used as the positive control (lane 1). Water was used for the no 'template' control (lane 2). The size marker lanes are shown in lanes 5 and 9 and GAPDH primers were used as an internal control to monitor the quality of RNA preparation (lanes 6–8). (b) Detection of P2XRs in a mixed population of anterior pituitary cells (lane 1) and immortalized GH₃ cells (lane 4). Plasmids containing cDNAs encoding P2XR were used as positive controls (lanes 2 and 5). For negative controls, PCR was conducted on first-strand cDNA samples without RT (lanes 3 and 6). (c) Expression of mRNA encoding P2X₂R in enriched somatotrophs (lane 2) and lactotrophs (lane 4). Plasmids containing cDNAs encoding P2X₂R were used as positive controls (lane 1). As a negative control, PCR was conducted on first-strand cDNA samples without RT (lane 3). The size marker lanes are shown in lanes 5 and 6. (d) Expression of mRNA encoding P2X₃R, P2X₄R and P2X₇R in enriched somatotrophs (lane 2) and lactotrophs (lane 4). Plasmids containing cDNAs encoding P2XR were used as positive controls (lane 1). For negative controls, PCR was conducted on first-strand cDNA samples without RT (lanes 3 and 5). Water was used for no 'template' controls (lane 6). The size marker lanes are shown in lanes 7 and 8 and GAPDH primers were used as an internal control to monitor the quality of RNA preparation (bottom panel). Abbreviation: GAPDH, glyceraldehyde phosphate dehydrogenase.

ATP ($\alpha\beta$ -meATP) and 2-methylthio-ATP (2-MeS-ATP), are also ineffective at generating Ca²⁺ signals in lactotrophs bathed in a Ca²⁺-deficient medium^{24,32}. Such a pharmacological profile is consistent with the expression of only the Y₂ subtype of P2YR in lactotrophs.

Expression of P2XR transcripts in anterior pituitary cells
Molecular identification of P2XRs expressed in anterior pituitary cells was also obtained by RT-PCR analysis²⁴. With the use of specific rat P2XR primers, transcripts encoding P2X₂R, P2X₃R, P2X₄R and P2X₇R were detected in a mixed population of

anterior pituitary cells (Fig. 2b, lane 1). Immortalized GH₃ pituitary cells also expressed transcripts for P2X₃R, P2X₄R and P2X₇R, but not P2X₂R (Fig. 2b, lane 4). In parallel with the GH₃ transcript profile, messages for P2X₃R, P2X₄R and P2X₇R were found in purified lactotrophs (Fig. 2d, lane 4), but not in somatotrophs (Fig. 2b, lane 2). By contrast, the message for P2X₂R was identified in somatotrophs (Fig. 2c, lane 2) but not in lactotrophs (Fig. 2c, lane 4). Finally, transcripts for P2X₁R, P2X₅R and P2X₆R were not present in mixed pituitary cells (Fig. 2b, lane 1) and GH₃ cells (Fig. 2b, lane 4).

Abundant PCR products of two different sizes, approximately 1.6 and 1.4 kb long, were consistently amplified by the P2X₂R primers in mixed pituitary cells and in purified somatotrophs, suggesting that two distinct lengths of transcripts for P2X₂R, with the same primer-annealing profiles, were expressed (Fig. 3a, lane 3). To test whether alternative splicing of the primary transcript of the gene encoding P2X₂R accounts for the occurrence of these two bands, the PCR products were purified separately from the gel and subcloned into a cloning vector. Restriction endonuclease mapping and nucleotide sequence analysis of the subcloned fragments showed the expression of the wild-type channel, denoted P2X_{2a}R, in the upper band and the spliced channel, denoted P2X_{2b}R, in the lower band (Fig. 3a). Several additional clones were also detected³³. The expression of spliced forms of P2X₂R is not unique to the pituitary – it is also seen in rat brain, kidney, spleen and intestine^{34,35}. In all these tissues, nucleotide sequence analysis revealed that P2X_{2b}R lacks a stretch of C-terminal amino acids (Val³⁷⁰–Gln⁴³⁸).

Signaling by recombinant P2XRs

We also compared the ability of recombinant P2XRs to initiate and sustain current and Ca²⁺ signaling. To do this, we expressed recombinant P2XRs in immortalized gonadotropin-releasing hormone (GnRH)-secreting GT1-7 neurons (hereafter known as GT1 cells). Similar to anterior pituitary cells and many other neuroendocrine and endocrine cells, GT1 cells exhibit spontaneous action potential-driven Ca²⁺ entry through T- and L-type voltage-gated Ca²⁺ channels. Furthermore, neither P2XRs nor the Ca²⁺-mobilizing P2Y receptors are native to GT1 cells³³, in contrast to many other immortalized cells that are commonly used for transfection studies. Therefore, these cells are an excellent mammalian model system to analyze Ca²⁺ signaling by P2XRs and its dependence on voltage-sensitive Ca²⁺ influx.

Because of the low sensitivity of P2X₇R to the native agonists, BzATP, a potent agonist analog for this receptor was used, whereas cells expressing P2X_{2a}R, P2X_{2b}R, P2X₃R and P2X₄R were stimulated with ATP. Figure 3b,c summarizes these investigations. Channel activation is rapid in cells expressing P2X_{2a}R and P2X_{2b}R. By contrast, there is a consistent difference in the pattern of desensitization

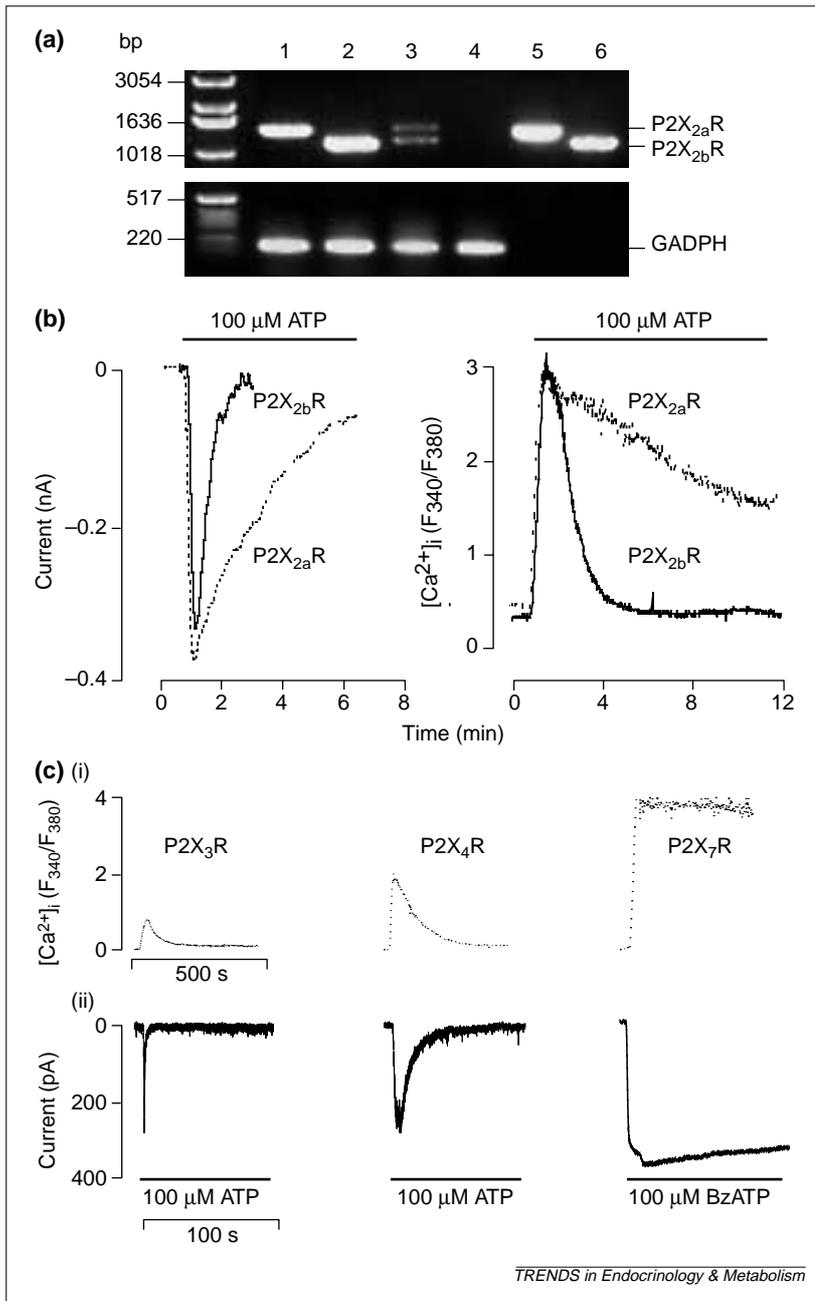


Fig. 3. Characterization of ion-conducting purinergic receptors (P2XRs) expressed in pituitary cells. (a) Expression of P2X₂R variants: RT-PCR analysis of total RNA from primary cultures of mixed pituitary cells (lane 3) and GT1 neurons expressing P2X_{2a}R (lane 1), P2X_{2b}R (lane 2) and without expression (lane 4); positive control of PCR for P2X_{2a}R (lane 5) and P2X_{2b}R (lane 6). Glyceraldehyde phosphate dehydrogenase (GADPH) primers were used as an internal control to monitor the quality of RNA preparation (lanes 1-4). (b) Pattern of (i) current signals and (ii) Ca²⁺ by recombinant P2X_{2a}R (dotted lines) and P2X_{2b}R (solid lines). (c) Pattern of (i) Ca²⁺ and (ii) current signals in GT1 cells expressing recombinant P2X₃R, P2X₄R and P2X₇R.

between the two channels. P2X_{2b}R desensitizes rapidly and completely during continuous stimulation with ATP, whereas P2X_{2a}R desensitizes slowly and incompletely. P2X₃R desensitizes more rapidly than P2X₄R, whereas no obvious desensitization was observed in P2X₇R-expressing cells stimulated with 100 μM BzATP for at least 10 min (Fig. 3c).

In summary, the following order reflects the desensitization rates of these five receptors:

P2X₃R > P2X_{2b}R = P2X₄R > P2X_{2a}R > P2X₇R. The rates of desensitization, but not the amplitude of current and [Ca²⁺]_i responses, are independent of the level of receptor expression. In cells expressing comparable levels of receptors, P2X₇R-expressing cells generate the highest amplitude of current and [Ca²⁺]_i responses, followed by cells expressing P2X_{2a}R, P2X_{2b}R, P2X₄R and P2X₃R (Ref. 36). These results indicate that expression of subtype-specific P2XRs provides an effective mechanism for generating variable current and [Ca²⁺]_i patterns in response to a common agonist.

Mechanism of P2X₂R desensitization

The lack of the Val³⁷⁰-Gln⁴³⁸ C-terminal amino acid sequence of the P2X_{2a}R molecule in P2X_{2b}R and the rapid desensitization of these channels³³⁻³⁵ suggest the importance of the spliced segment to prolonged Ca²⁺ influx through wild-type channels. We attempted to identify the structural elements in the P2X_{2a}R C-terminus that are responsible for prolonged opening of channels. Initially, a series of C-terminal truncated mutants was produced to narrow the region(s) needed for the slow desensitization pattern of P2X_{2a}R. Subsequently, triple Ala replacement and single amino acid deletion mutants were constructed to identify precisely the amino acid sequence that is crucial for long-lasting Ca²⁺ signaling by P2X_{2a}R. Finally, spliced amino acids from the C-terminus of the wild-type channel were gradually added to the P2X_{2b}R to regain the slow desensitizing pattern of Ca²⁺ signaling in response to prolonged agonist stimulation. The results of this investigation (discussed in detail in Ref. 37) indicate that a polypeptide region containing Arg³⁷¹-Pro³⁷⁶ residues is the sequence important for the prolonged Ca²⁺ influx of P2X_{2a}R.

This six amino acid region is located near the second putative transmembrane domain and the difference in the C-terminal sequences among the members of P2XRs begins at this region^{14,38}. To study the possible role of the structural diversity of this region in the control of receptor desensitization, the Arg³⁷¹-Pro³⁷⁶ sequence of P2X_{2a}R was introduced into P2X₃R and P2X₄R instead of the native Thr³⁶²-Lys³⁶⁷ and Glu³⁷⁶-Gly³⁸¹ sequences, respectively. The opposite mutations were also performed at the P2X_{2a}R C-terminus by substituting the Arg³⁷¹-Pro³⁷⁶ sequence with the corresponding six amino acid sequences of P2X₃R, P2X₄R and P2X₁R. In cells expressing mutant P2X₃R or P2X₄R, the desensitization rate of the ATP-induced [Ca²⁺]_i response was slower than that of wild-type receptors. By contrast, all chimeric P2X_{2a}R subunits substituted at C-terminal six amino acids formed functional channels in GT1 cells that exhibited enhanced desensitization rates when compared with the wild-type P2X_{2a}R (Ref. 39). These results indicate that the Arg³⁷¹-Pro³⁷⁶ sequence of P2X_{2a}R and the equivalent sequences of P2X₃R and P2X₄R are important in determining the desensitization to ATP stimulation.

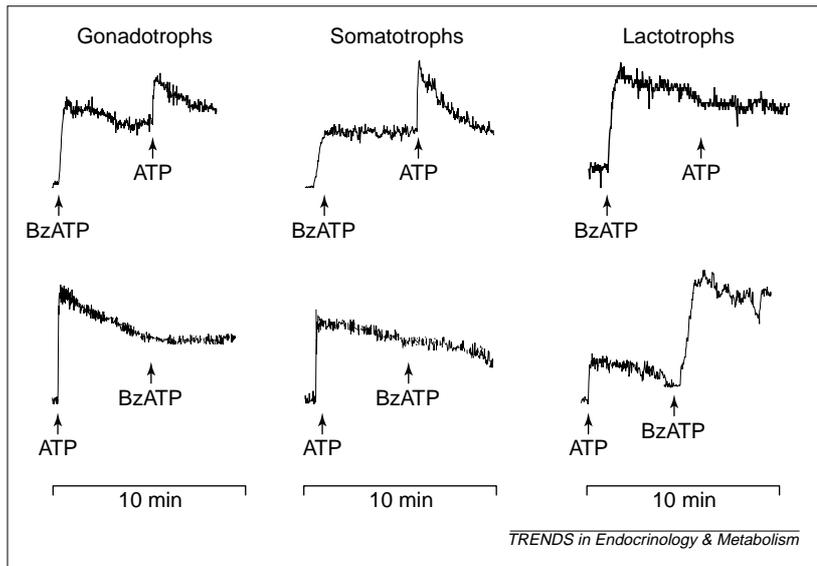


Fig. 4. Pharmacological characterization of ion-conducting purinergic receptors (P2XRs) expressed in anterior pituitary cells. Typical pattern of Ca^{2+} signals in gonadotrophs, somatotrophs and lactotrophs stimulated with ATP and its analog, BzATP [3'-O-(4-benzoyl)benzoyl-ATP].

Identification of P2XRs expressed in pituitary cells

In addition to differences in the amplitude of current and $[Ca^{2+}]_i$ signals and the rates of receptor desensitization, recombinant P2XRs also differ in their sensitivity to ATP analog agonists. For example, P2X₃Rs are sensitive to $\alpha\beta$ -meATP (Ref. 39), whereas P2X_{2a}R, P2X_{2b}R, P2X₄R and P2X₇R are not^{24,40}. Furthermore, homomeric P2X_{2a}R, P2X_{2b}R and P2X₇R are sensitive to the P2XR antagonist suramin, whereas P2X₄R is not². Finally, P2X₇R is highly sensitive to BzATP and less sensitive to ATP, whereas P2X_{2a}R and P2X_{2b}R are more sensitive to ATP than BzATP (Refs 24,36). We used these receptor-specific current and $[Ca^{2+}]_i$ and pharmacological profiles as effective tools in searching for cell-specific expression of P2XRs among anterior pituitary cells.

$\alpha\beta$ -meATP was able to induce a rise in $[Ca^{2+}]_i$ in only 50% of lactotrophs, the pattern of which was highly comparable to that observed in cells expressing recombinant P2X₃R. In ~33% of lactotrophs, ATP induced a suramin-insensitive $[Ca^{2+}]_i$ response, the pattern of which was indistinguishable from that observed in cells expressing recombinant P2X₄R. Furthermore, BzATP, an agonist that activates P2X₇R and P2X₂R, induced rises in $[Ca^{2+}]_i$ in most lactotrophs, somatotrophs and gonadotrophs. Approximately 75% of lactotrophs responded to BzATP with the non-desensitizing $[Ca^{2+}]_i$ response typically seen in P2X₇R-expressing cells, and addition of ATP in the presence of BzATP had no effect (Fig. 4, right-hand tracings). By contrast, addition of ATP in the presence of BzATP further increased $[Ca^{2+}]_i$ in gonadotrophs and somatotrophs (Fig. 4), indicating that these cells express the P2X₂R subtype. These results, summarized in Table 1, agree with RT-PCR analysis (Fig. 2), confirming that lactotrophs express P2X₃R, P2X₄R and P2X₇R, as well as P2Y₂R, whereas

gonadotrophs and somatotrophs express P2X₂R exclusively²⁴.

The coexpression of P2XRs in single lactotrophs was seen frequently. A fraction of cells expressing the $\alpha\beta$ -meATP-sensitive P2X₃Rs also responded to the application of BzATP and ATP with an additional rise in $[Ca^{2+}]_i$, presumably by activating P2X₇R. Similarly, ~15% of lactotrophs responded to BzATP with a non-desensitizing response, and ATP with a spike response, suggesting the expression of both P2X₇R and P2Y₂R in the same cells. Finally, ATP was able to induce a suramin-insensitive rise in $[Ca^{2+}]_i$ in a small fraction of lactotrophs already stimulated with BzATP, the profile of which was comparable to that observed in GT1 cells expressing P2X₄R (Ref. 24).

Heteropolymerization of P2XRs

The assembly of functional P2XRs requires three or four subunits, and the native channels are either homomeric or heteromeric. Heteropolymerization leads to distinct pharmacological profiles, amplitudes of current and $[Ca^{2+}]_i$ responses and kinetics of receptor activation and desensitization. The number and type of subunits expressed in a particular cell determine the nature of heteromers. In addition, not all P2XR subunits can generate functional heteromeric channels. P2X₅R and P2X₆R frequently co-assemble with P2X₁R, P2X₂R and P2X₃R; P2X₃R subunits, P2X₂R and P2X₁R subunits co-assemble in all combinations; whereas P2X₄R and P2X₇R form only homomeric channels⁴¹. In accordance with these observations, the coexpression of P2X₃R, P2X₄R and P2X₇R in GT1 neurons in any combination does not generate functional heteromers. Furthermore, the kinetics of current and $[Ca^{2+}]_i$ signals generated by pituitary P2X₃R, P2X₄R and P2X₇R expressed in lactotrophs are highly comparable to those observed in cells expressing homomeric recombinant channels, suggesting that native channels in lactotrophs are also homomers^{24,36}.

Consistent with other data^{12,13}, cotransfection of P2X_{2a}R and P2X₃R into GT1 neurons results in channels that respond to $\alpha\beta$ -meATP (P2X₃R-like), but with a higher amplitude of $[Ca^{2+}]_i$ response (P2X_{2a}R-like). These channels desensitize more rapidly than the homomeric P2X_{2a}R, but slower than P2X₃R, an additional feature that suggests that the $\alpha\beta$ -meATP-mediated rise in $[Ca^{2+}]_i$ is initiated by activation of heteromeric P2X_{2a}R–P2X₃R (Ref. 39). However, none of the $\alpha\beta$ -meATP-sensitive pituitary

Table 1. Distribution of functional P2XRs in pituitary cells^a

Cell type	P2X ₂ R	P2X ₃ R	P2X ₄ R	P2X ₇ R
Gonadotrophs	84	0	0	0
Somatotrophs	82	0	0	0
Lactotrophs	0	50	33	74

^aNumbers given are as a percentage of pituitary cells examined.

cells generated $[Ca^{2+}]_i$ signals with an amplitude and duration comparable to that observed in GT1 cells bearing heteromeric P2X₂R–P2X₃R. This is consistent with the absence of P2X₂R transcripts from lactotrophs, and P2X₃R transcripts from somatotrophs (Fig. 2) and gonadotrophs (not shown), indicating that the functional P2X₂R–P2X₃R heteromers are not native to pituitary cells.

By contrast, gonadotrophs and somatotrophs frequently express P2X₂Rs with distinct Ca^{2+} signal profiles; that is, the rates of desensitization are in the range between those observed in cells expressing recombinant P2X_{2a}R and P2X_{2b}R (Refs 33,42). These results indicate that heteropolymerization might account for the differences observed between native and cloned channels. To test this hypothesis, GT1 neurons were transfected with cDNAs encoding both P2X_{2a}R and P2X_{2b}R in different ratios, keeping the total amount of transfected plasmids unchanged. The pattern of ATP-elicited Ca^{2+} signaling was between that of P2X_{2a}R and P2X_{2b}R, and comparable to that observed in pituitary cells³³. The coexpression of P2X_{2a}R and P2X_{2b}R probably provides an effective mechanism for controlling extensive Ca^{2+} influx during sustained receptor activation in somatotrophs and gonadotrophs.

P2XRs and voltage-sensitive and -insensitive Ca^{2+} influx

In excitable cells, activated P2XRs generate Ca^{2+} signals by two mechanisms: directly, by conducting Ca^{2+} (in addition to other ions), and indirectly, by depolarizing cells and facilitating voltage-sensitive Ca^{2+} influx. To examine the extent to which voltage-sensitive Ca^{2+} influx participates in P2XR-generated Ca^{2+} signals, experiments were carried out in both pituitary cells and GT1 neurons expressing recombinant P2X_{2a}R, P2X_{2b}R, P2X₃R, P2X₄R and P2X₇R. In one set of experiments, cells were clamped at -85 mV to exclude voltage-sensitive Ca^{2+} influx, and in the other set of experiments cells were bathed in a medium containing nifedipine, a voltage-gated Ca^{2+} channel blocker^{24,36,42}. Both experiments indicate that Ca^{2+} signaling by all P2XRs is to some extent dependent on both voltage-insensitive and voltage-sensitive Ca^{2+} influx.

Ca^{2+} signaling by recombinant P2X₃R and P2X₄R is predominantly dependent on voltage-sensitive Ca^{2+} influx, whereas both pathways equally contribute to $[Ca^{2+}]_i$ responses in cells expressing P2X_{2a}R and P2X_{2b}R. The activation of P2X₇R also facilitates voltage-sensitive Ca^{2+} influx, but only transiently and when cells are stimulated with lower ATP concentrations. The inefficacy of nifedipine at supramaximal concentrations and the high amplitude of current and $[Ca^{2+}]_i$ responses further indicate that this channel operates as a nonselective pore capable of conducting larger amounts of Ca^{2+} independently of the status of voltage-gated Ca^{2+} channels. Finally, a prolonged stimulation of P2X₇R-expressing GT1 neurons with $100 \mu M$ BzATP

leads to permeabilization of plasma membrane in a fraction of cells. In other cell types, it has been shown that this activation step is dependent on the presence of the C-terminus of the receptor¹¹ and on the environmental temperature⁴³. Because the increased permeability results in larger ion fluxes and leakage of small metabolites, it might cause cell swelling and vacuolization, leading to cell death by necrosis and/or apoptosis². However, it is premature to discuss the possible role of P2X₇R in the control of cell death in lactotrophs. Finally, activated voltage-gated Ca^{2+} channels also undergo inactivation in a $[Ca^{2+}]_i$ -dependent manner⁴⁴, but the time needed for the development of this inactivation is longer than the rates of P2X₁R, P2X_{2a}R, P2X_{2b}R, P2X₃R and P2X₅R desensitization. In accordance with this, no change in the rates of P2X_{2a}R and P2X_{2b}R desensitization was observed in cells bathed in the presence of nifedipine³⁹.

Physiological relevance of purinergic receptors

By contrast to well characterized Ca^{2+} signaling by P2 receptors, very limited information is available regarding their possible physiological and pathophysiological relevance. Pituitary cells store and secrete ATP during agonist-induced exocytosis^{31,42,44}, suggesting that this compound acts as an autocrine–paracrine factor. The addition of ATP leads to the stimulation of basal PRL and gonadotropin secretion and amplification of TRH- and GnRH-induced hormone secretion^{31,42,44}. At the present time, there is no information about the P2 receptor subtypes involved in the secretory pathways and the mode of receptor interaction in cells expressing multiple receptor subtypes. We believe that ATP acts locally on cells expressing P2XRs as an amplifier of Ca^{2+} signaling and secretion, by promoting voltage-sensitive and -insensitive Ca^{2+} influx. P2YRs might also have some additional functions in pituitary cells, comparable to those observed in other cell types^{27,28}. Recently, published data also demonstrated the expression of P2X₂R in human pituitaries⁴⁵, indicating that the purinergic control of pituitary functions is not unique to rodents.

Concluding remarks

Pituitary cells secrete ATP and its extracellular messenger actions are controlled by ecto-ATPases, the set of enzymes that degrade ATP to ADP, AMP and adenosine. In addition to ATP, ADP and adenosine also have the potential to operate as ligands for purinergic receptors. RT-PCR and single cell physiological studies have indicated the expression of P2Y₂R, P2X_{2a}R, P2X_{2b}R, P2X₃R, P2X₄R and P2X₇R in anterior pituitary cells. Pharmacological and secretory studies have also suggested the expression of A₁Rs. Purinergic receptors have a dual action on Ca^{2+} signaling: stimulatory in cells expressing P2Rs, and inhibitory in cells expressing A₁R. The specificity of P2R actions is achieved by the capacity of

individual receptor subtypes to generate different amplitudes and kinetic patterns of Ca^{2+} responses, and by the selective expression of receptors within pituitary cell subpopulations.

P2XRs are expressed in all five major secretory cell types. Further study into the identification of P2XR subtype(s) expressed in thyrotrophs and corticotrophs is required. The ATP signaling pathway in somatotrophs is relatively simple. Most of these cells express P2X_{2a}R and P2X_{2b}R. Molecular cloning and expression of the rat gene encoding P2X₂R revealed two physiologically important characteristics: a relatively high Ca^{2+} permeability and an efficient mechanism to protect the cell from overloading with Ca^{2+} . The control is achieved by alternative splicing of the gene encoding P2X₂R and coexpression of wild-type and spliced channels. The charged residues in the six amino acid C-terminal sequence appear to serve as a common factor influencing the desensitization rates of several ATP-gated channels in addition to P2X_{2a}R.

Our results with gonadotrophs from Sprague–Dawley rats also indicate the expression of P2X₂Rs, but not P2Y₂R, in these cells. In line with this, Tomic *et al.*⁴² and Billiard⁴⁶ found that gonadotrophs from Sprague–Dawley rats respond to ATP with a depolarizing current typically observed in P2X₂R-expressing cells, but not with periodic hyperpolarizing currents typical for GnRH- and endothelin-stimulated gonadotrophs. By contrast, gonadotrophs of Wistar rats express functional P2Y₂Rs (Ref. 25). Further studies are required to clarify what underlies this difference: the strain of

animals, dispersion and cultural conditions or some other factor(s). In addition to the majority of other cell lines studied^{26,27,33}, immortalized mouse α 3T-1 gonadotrophs express functional P2Y₂R (Ref. 28), suggesting that immortalization *per se* might lead to the expression of these receptors.

The most striking finding is the complexity of the purinergic signaling system of lactotrophs. These cells express three types of channel (P2X₃R, P2X₄R and P2X₇R) in addition to G-protein-coupled P2Y₂R. Others have also reported on the expression of P2Y₂R in lactotrophs³² and P2X₇R in GH₃ cells⁴⁷. When activated, P2XRs generate $[Ca^{2+}]_i$ signals of different amplitude and duration. P2X₃R generates small-amplitude $[Ca^{2+}]_i$ signals, which desensitize within 1 to 2 min. The amplitude and the profile of Ca^{2+} signals generated by P2X₄R are comparable to those observed in cells expressing P2X_{2b}R. By contrast, P2X₇R generates high-amplitude $[Ca^{2+}]_i$ signals that do not desensitize during the prolonged agonist stimulation. Ca^{2+} signaling by P2X₃R and P2X₄R is predominantly dependent on voltage-sensitive Ca^{2+} influx, whereas P2X₇R operates as a non-selective pore capable of conducting larger amounts of Ca^{2+} independently of the status of voltage-gated Ca^{2+} channels. At the present time, there is no available information about the functional consequence and importance of the complexity of purinergic receptor expression in pituitary cells, the heterogeneity of $[Ca^{2+}]_i$ signals generated by ATP or the mode of interaction of P2Y₂R and P2XRs in cells coexpressing both types of receptor.

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Diabetes mellitus and diabetes-associated vascular disease

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Diabetes-related cardiovascular disease remains the leading cause of death in patients with type 2 diabetes. Hypertension is common among diabetics and has the same pathogenetic mechanisms as insulin resistance, in which the activated renin-angiotensin system contributes to the emerging high blood pressure and hyperglycemia. Hyperglycemia is one of the triggering factors for vascular dysfunction and clotting abnormalities and, therefore, for accelerated atherosclerosis in diabetes. Glycated hemoglobin levels, as a reflection of the degree of glycemia, are strongly associated with the risk of cardiovascular disease in diabetics and in the general population. Tight glycemic control, the treatment of dyslipidemia and raised blood pressure, in addition to the use of antiplatelet therapy, all powerfully reduce the risks associated with diabetes. Furthermore, angiotensin-converting enzyme inhibitors might offer additional cardioprotection to diabetics above that provided by blood pressure reduction.

Long-term vascular complications are the main cause of mortality and morbidity associated with diabetes mellitus and the insulin-resistant state^{1–4}. The risk of coronary artery disease (CAD) is increased two- to fourfold in diabetics, putting these patients in the same risk group as those who have survived a stroke or a myocardial infarction (MI) (Refs 2,3,5). Cardiovascular disease (CVD) accounts for up to 80% of deaths in patients with type 2 diabetes mellitus (T2DM) (Fig. 1). Moreover, diabetics are more likely to develop congestive heart failure (CHF) (Refs 6, 7), partly because of the association of CAD with diabetic

and hypertensive cardiomyopathy. The relative risk of stroke in patients with diabetes is two- to threefold higher than in nondiabetic individuals, and is higher in females than in males^{1,5}. Diabetes increases the prevalence of CVD in premenopausal women, negating the cardioprotective effects that estrogen normally provides to women before the menopause^{3,6}. Furthermore, diabetic women are much more likely to die of MI than nondiabetic women⁸. In general, diabetes mellitus in women is associated with older age, obesity, hypertension, higher triglyceride levels and lower levels of high-density lipoproteins (HDL) (Ref. 2), which are the same factors that cause the development of CAD in the general population. Furthermore, hyperglycemia, platelet hyperaggregability and/or platelet adhesiveness and coagulation abnormalities, in addition to associated hypertension, all contribute to the accelerated atherosclerotic process seen in T2DM.

T2DM and hypertension

Hypertension occurs more frequently in diabetics than in the nondiabetic population^{9,10}, contributing to the high morbidity and mortality in patients with diabetes. It has been estimated that hypertension contributes to 35–75% of diabetic complications¹¹.

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