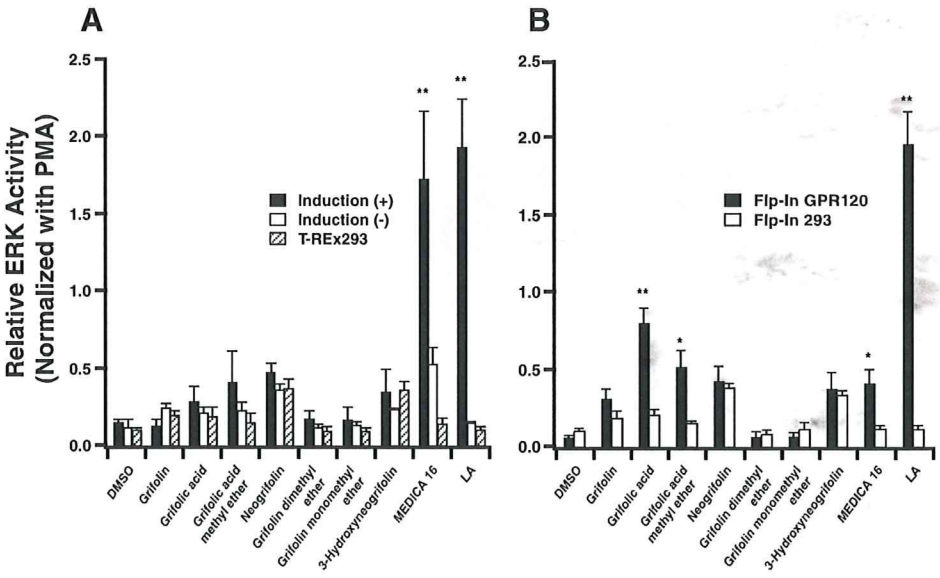

Fig. 2 Effects of each compound on ERK activation in T-REx GPR40 and Flp-in GPR120 cells. T-REx GPR40 cells that had been incubated with Dox [induction (+)] or without Dox [induction (−)] and T-REx 293 cells (**a**) and Flp-in GPR120 cells or Flp-in 293 cells (**b**) were stimulated with each compound at 100 μM final concentration. Cell lysates were analyzed by Western blotting using anti-phospho- and anti-total-kinase antibodies. Data are means±SE of three independent experiments. *$P<0.05$ and **$P<0.01$ vs. DMSO

with each compound that was being tested at a concentration of 100 μM. After 10 min of incubation with each compound, total cell extracts were prepared and subjected to Western blotting using anti-phospho- and anti-total-kinase antibodies.

GLP-1 secretion

GLP-1 secretion from STC-1 cells was measured as described previously (Hirasawa et al. 2005). Briefly, STC-1 cells were seeded at a density of 4×10^5 cells/well on 24-well plates, incubated for 48 h at 37°C in the medium, and stimulated with compounds in HBSS for 1 h at 37°C. After incubation, supernatants were collected, and the concentration of GLP-1 was determined by enzyme immunoassay with Rat GLP-1 ELISA Kit Wako (Wako, Osaka, Japan).

Statistical analysis

The level of significance for the difference between sets of data was assessed using an unpaired Student's *t* test. Data were expressed as means±SE. $p<0.05$ was considered as statistically significant.

Results

Analysis of cell lines expressing GPR40 and GPR120

In order to identify novel ligands for GPR40 and GPR120, a Dox-inducible cell line expressing hGPR40 (T-REx GPR40) and a stable cell line expressing hGPR120 (Flp-in GPR120) were developed as described in "Materials and

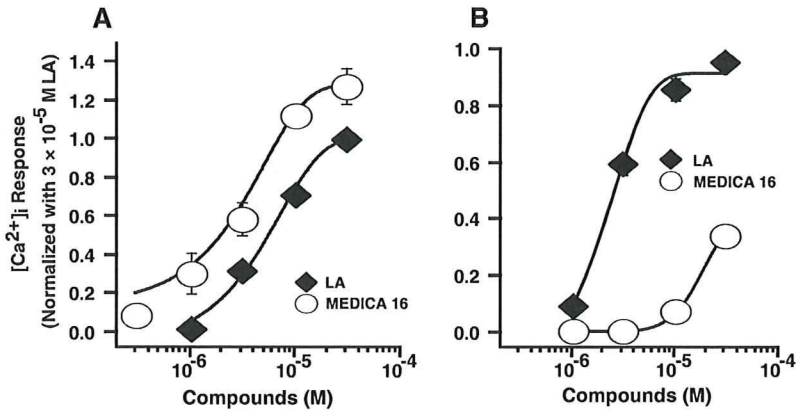

Fig. 3 Effects of MEDICA16 on $[Ca^{2+}]_i$ mobilization in T-REx GPR40 and Flp-in GPR120 cells. T-REx GPR40 cells that had been incubated with Dox [induction (+)] (**a**) and Flp-in GPR120 cells (**b**) were stimulated with various concentrations of LA or MEDICA16

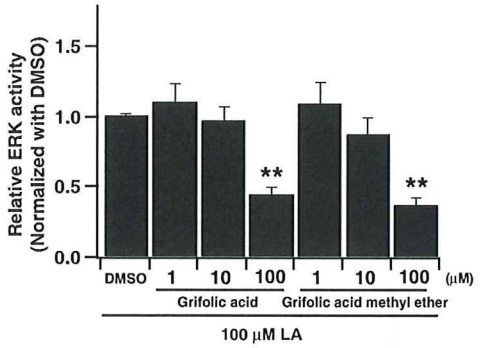

Fig. 4 Inhibition effects of test compounds on ERK activation in Flp-in GPR120 cells. Flp-in GPR120 cells were stimulated with LA at 100 μM final concentration. After stimulation with LA, cells were loaded for 5 min with test compounds at the indicated concentrations. Data are means±SE of three independent experiments. $^{**}P<0.01$ vs. DMSO

methods". To determine whether these two receptors possess the agonist responsiveness, ERK and $[Ca^{2+}]_i$ responses were measured after stimulation of these cell lines with LA, which is known to activate GPR40 and GPR120 (Briscoe et al. 2003; Itoh et al. 2003; Hirasawa et al. 2005). LA increased ERK and $[Ca^{2+}]_i$ responses in a dose-dependent manner in T-REx GPR40 cells incubated with Dox [Induction (+); Fig. 1a, (a) and b, (a)] and in Flp-in GPR120 cells [Fig. 1a, (b) and b, (b)]; however, T-REx 293 cells, T-REx GPR40 cells incubated without Dox [induction (−)], and Flp-in 293 cells showed only a basal level of activation after addition of LA. These results indicated that the activation of ERK and $[Ca^{2+}]_i$ responses induced by LA in these cell lines was mediated by these receptors and was not a nonspecific effect.

Screening for the GPR40 and GRP120 ligands

Using the cell lines described herein, in order to identify selective ligands for GPR40 and GPR120, 80 natural compounds were screened, together with the reference compound MEDICA16, which is known to activate GPR40, by monitoring ERK activity in T-REx GPR40 cells and in Flp-in GPR120 cells. Among the 80 natural compounds, a series of grifolin derivatives isolated from plant materials (Table 1) was identified, which possess the ability to activate GPR120. As shown in Fig. 2a, none of the grifolin derivatives tested activated ERK in T-REx GPR40 cells incubated with Dox. As shown in Fig. 2b, the grifolin derivatives, grifolic acid and grifolic acid methyl ether, activated ERK in Flp-in GPR120 cells, although the potencies of these two compounds were much lower than that of LA. MEDICA16 increased the ERK activity both in T-REx GPR40 cells incubated with Dox and in Flp-in GPR120 cells; however, the effect of this compound on ERK activity was more potent in the T-REx GPR40 cells (Fig. 2a) compared to the Flp-in GPR120 cells (Fig. 2b). Thus, the screens identified specific ligands for GPR40 and GPR120.

Pharmacological effects of the ligands on GPR40 and GPR120

The effects of MEDICA16, grifolic acid, and grifolic acid methyl ether on activation of GPR40 and GPR120 were next examined in greater detail. In accordance with the result of the ERK assay, MEDICA16 potently increased $[Ca^{2+}]_i$ in a dose-dependent manner in T-REx GPR40 cells incubated with Dox, compared to the response in Flp-in GPR120 cells (Fig. 3a, b), indicating that MEDICA16 could be a selective agonist for GPR40.

As shown in Fig. 2b, some grifolin derivatives activated GPR120, but their potency was far less than that of LA. Thus, we speculated that they might act as partial agonists for GPR120, so we next examined whether grifolic acid and grifolic acid methyl ether could act as antagonists for

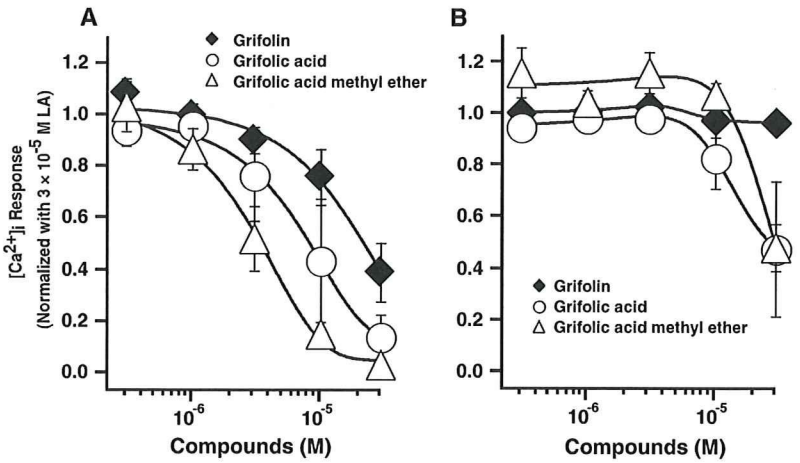

Fig. 5 Inhibition curves of $[Ca^{2+}]_i$ response induced by LA with test compounds in Flp-in GPR120 or T-REx GPR40 cells. Flp-in GPR120 (**a**) and T-REx GPR40 cells (**b**) were stimulated with LA at 100 μM final concentration. After stimulation with LA, test compounds were loaded at the indicated concentrations. Data are means ±SE of three independent experiments

Table 2 Comparison of apparent pA_2 and relative affinity for grifolic acid and grifolic methyl ether

Antagonist	Number of experiments	Apparent pA_2 ($\times 10^{-6}$M)	Relative affinity
Grifolic acid	3	4.95±0.1	1.00[a]
Grifolic acid methyl ether	3	5.01±0.1	0.79 NS

Apparent K_B values were converted to apparent pA_2 values as described in "Materials and methods". Results are means±SE of three independent experiments

NS not significant

[a] Grifolic acid, arbitrary set at 1

GPR120. As shown in Fig. 4, these two compounds inhibited LA-induced ERK phosphorylation in a dose-dependent manner in Flp-in GPR120 cells. In addition, these compounds together with grifolin inhibited LA-induced $[Ca^{2+}]_i$ in a dose-dependent manner. On the other hand, grifolic acid and grifolic acid methyl ether inhibited LA-induced $[Ca^{2+}]_i$ in T-REx GPR40 cells incubated with Dox, but their inhibitory effects were less than that seen in Flp-in GPR120 cells (Fig. 5a, b). In addition, the pA_2 values of these two compounds for GPR120 were calculated as described in "Materials and methods". The relative affinity of grifolic acid methyl ether and that of grifolic acid was compared by dividing the apparent K_B of grifolic acid methyl ether by that of grifolic acid (Table 2). This result revealed that the antagonistic effects of these two compounds were not significantly different. Other grifolin derivatives, specifically neogrifolin, grifolin dimethyl ether, grifolin monomethyl ether, and 3-hydroxyneogrifolin, did not inhibit ERK or $[Ca^{2+}]_i$ responses in the two cell lines (data not shown). These results indicated that some grifolin derivatives could be selective partial agonists for GPR120.

The biological effects of grifolic acid and grifolic acid methyl ether on GPR120 were next examined by measuring GLP-1 secretion from STC-1 cells. Stimulation of GPR120 has been shown to promote secretion of GLP-1 from STC-1 cells (Hirasawa et al. 2005). Therefore, the effects of these compounds on GLP-1 secretion from STC-1 cells were examined. As shown in Fig. 6, LA, grifolic acid, and grifolic acid methyl ether stimulated GLP-1 secretion in a dose-dependent manner. On the other hand, MEDICA16 stimulation did not increase GLP-1 secretion significantly. Thus, the effects of these compounds on GPR120 activation in Flp-in GPR120 cells correlated well with their ability to induce GLP-1 secretion from STC-1 cells via GPR120.

Discussion

This report identifies and characterizes selective ligands for GPR40 and GPR120 that were isolated from natural compounds. The compounds, together with the reference compound MEDICA16, were identified by measuring intracellular signals ($[Ca^{2+}]_i$ and ERK responses) in inducible and stable expression cell lines. These cell lines were established previously, and the expression and function of these receptors were examined (Hirasawa et al. 2005, 2008b; Hara et al. 2009), leading to the development of assays that could discriminate whether signals induced by various ligands were mediated by specific receptors. This led to identification of the selective GPR40 ligand MEDICA16 and the selective GPR120 ligands grifolic acid and grifolic acid methyl ether.

MEDICA16 is known to activate GPR40 (Kotarsky et al. 2003), and it is an experimental anti-obesity compound (Bar-Tana et al. 1985). However, the selectivity of MEDICA16 between GPR40 and GPR120 has not yet been elucidated in the literature. In this study, the effect of MEDICA16 on GPR40 activation was found to be more potent than its effect on GPR120. Hence, MEDICA16 may act as a selective agonist that could be used as a selective pharmacological probe for GPR40. Moreover, the results of screening 80 natural compounds demonstrated that two grifolin derivatives, grifolic acid and grifolic acid methyl

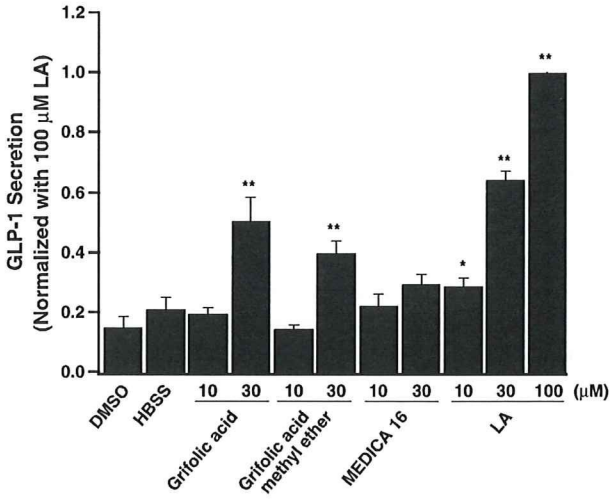

Fig. 6 Effects of test compounds on GLP-1 secretion in STC-1 cells. STC-1 cells were incubated with the test compounds for 60 min at 37° C in HBSS (30 μM). Data are means±SE of three or four independent experiments. *$P<0.05$ and **$P<0.01$ vs. DMSO

ether, which were isolated from the fresh fruiting bodies of the mushroom *A. confluens* or were synthesized with chemical modification of these compounds, possessed pharmacological activity by acting through GPR120. This study confirms that grifolic acid and grifolic acid methyl ether not only activated ERK and $[Ca^{2+}]_i$ responses through GPR120 signaling, but they also inhibited responses induced by LA in GPR120-expressing cells. Therefore, these two compounds may act as novel partial agonists for GPR120. In addition, they may also act as antagonists for GPR40, but their potencies as antagonists were much less pronounced. These two compounds showed both agonistic and antagonistic activities when used to stimulate GPR120-expressing cells. However, additional grifolin derivatives (neogrifolin, grifolin dimethyl ether, grifolin monomethyl ether, and 3-hydroxyneogrifolin) did not induce any responses in either receptor. In accordance with previous reports (Itoh et al. 2003; Hirasawa et al. 2005), these results also indicated that the carboxyl group of grifolin derivatives was indispensable for the activation of GPR120. In addition, although further investigations of GPR120 ligand selectivity for GPR120 are needed, these results provide useful information on structure–activity relationships between GPR120 and its ligands.

The biological properties of these compounds on GLP-1 secretion via GPR120 were further investigated using STC-1 cells, which express GPR120 endogenously. Their effects on GLP-1 cells correlated well with their effect on $[Ca^{2+}]_i$ and ERK responses in Flp-in GPR120 cells. In contrast, MEDICA16, which selectively activated ERK and $[Ca^{2+}]_i$ response in T-REx GPR40 cells, did not induce GLP-1 secretion from STC-1 cells. These results indicated that grifolic acid and grifolic acid methyl ether could be useful probes with which to discriminate between the pharmacological effects of GPR40 and GPR120.

Grifolin derivatives have been shown to possess a broad spectrum of biological effects, such as inhibiting growth and inducting significant apoptosis in some cancer cell lines (Ishii et al. 1988; Zechlin et al. 1981; Sugiyama et al. 1992). They also showed hypocholesterolemic action in rats fed a high-cholesterol diet (Sugiyama et al. 1992). However, the relationship between GPR120 and grifolin derivatives has not been reported. Hence, the effect of these compounds on GLP-1 secretion via GPR120 represents a novel biological property of grifolin derivatives.

Recently, the synthetic 4-(benzylamino)dihydrocinnamic acid derivative (GW9508) and the 4-phenethynyldihydrocinnamic acid derivative (TUG-424) were shown to possess agonistic activity towards GPR40 (Briscoe et al. 2006; Christiansen et al. 2008). GW9508 showed agonistic activity towards GPR120 and GPR40. On the other hand, only the effect on GPR40 has been examined for TUG-424. In addition, 4-{4-[2-(phenyl-2-pyridinylamino)ethoxy]phenyl}butyric acid (compound 12) have been identified as selective ligands for GPR120, but their selectivity between GPR40 and GPR120 was evaluated only using an overexpression system (Suzuki et al. 2008). Thus, the selectivity of various ligands between GPR40 and GPR120 remains to be fully elucidated. At present, the pharmacology of FFARs, including GPR40 and GPR120, is not fully understood despite intensive research efforts, mainly because there are few specific and selective ligands for GPR120. Thus, the compounds identified in this study could be used as novel selective probes capable of distinguishing pharmacological and physiological effects related to signaling through GPR40 and GPR120.

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Review

Signaling by purinergic receptors and channels in the pituitary gland

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ABSTRACT

Adenosine 5′-triphosphate is frequently released by cells and acts as an agonist for G protein-coupled P2Y receptors and ligand-gated P2X cationic channels in numerous tissues. The breakdown of ATP by ectonucleotidases not only terminates its extracellular messenger functions, but also provides a pathway for the generation of two additional agonists: adenosine 5′-diphosphate, acting via some P2Y receptors, and adenosine, a native agonist for G protein-coupled adenosine receptors. In the pituitary gland, adenosine 5′-triphosphate is released from the endings of magnocellular hypothalamic neurons and by anterior pituitary cells through pathway(s) that are still not well characterized. This gland also expresses several members of each family of purinergic receptors. P2X and adenosine receptors are co-expressed in the somata and nerve terminals of vasopressin-releasing neurons as well as in some secretory pituitary cells. P2X receptors stimulate electrical activity and modulate $InsP_3$-dependent calcium release from intracellular stores, whereas adenosine receptors terminate electrical activity. Calcium-mobilizing P2Y receptors are expressed in pituicytes, folliculo-stellate cells and some secretory cells of the anterior pituitary.

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Contents

1. Introduction

Purines adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP) and adenosine, and pyrimidines uridine-5′-triphosphate (UTP) and uridine-5′-diphosphate (UDP) are not only energy sources and important intracellular molecules that control various cellular processes, but they are also extracellular messengers. The concept of purinergic signaling was first proposed by Burnstock (1972). Since then, the extracellular roles of purines and pyrimidines have been established in numerous tissues, including the pituitary gland. The effects of these messengers are mediated via two main groups of purinergic receptors: G protein-coupled adenosine receptors (ARs), also known as P1 receptors, and ATP, ADP, UTP and/or UDP-activated P2 receptors (P2Rs). ARs are a single family of receptors, whereas P2Rs belong to one of two families: G protein-coupled receptors (P2YRs) and ligand-gated ion channels (P2XRs) (Ralevic and Burnstock, 1998).

The release of endogenous nucleotides represents a critical component for the activation of these receptors. The roles of ATP as a neurotransmitter or co-transmitter and adenosine as an important neuromodulator are well established in the peripheral and central nervous systems. Cardiac tissue releases adenosine, whereas ATP is secreted by skeletal muscle, adrenal chromaffin cells, pancreatic beta cells, mast cells, blood cells, fibroblasts, and endothelial cells in response to mechanical stress, hypoxia, acidosis, osmotic shock or inflammation (Bodin and Burnstock, 2001a,b). Several reports have also suggested that pyrimidines are secreted by nonexcitable cells,

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including endothelial, epithelial, and astrocytoma cells (Yegutkin, 2008). The duration and distance of ATP's actions are limited by several ectonucleotidases, which ensure that circulating levels of ATP are below that required for the global activation of purinergic receptors. The currently identified ectonucleotidases include members of the ectonucleotide triphosphate diphosphohydrolase family (eNTPDase) and several other subfamilies of enzymes. These enzymes not only hydrolyze extracellular ATP and/or ADP to adenosine 5′-monophosphate (AMP) and adenosine, but also metabolize other nucleotide tri- and diphosphates, including UTP and UDP (Yegutkin, 2008; Zimmermann, 2000).

Released ATP is a native agonist for P2XRs. Molecular, physiological, and pharmacological studies have revealed the existence of seven subunits, denoted as P2X1 through P2X7, as well as several spliced forms of these subunits. Each subunit is proposed to have two transmembrane helices connected by a large extracellular loop, with both N- and C-termini located in the cytoplasm. From the N-termini through the second transmembrane domain, the cloned subunits exhibit a relatively high level of amino acid sequence homology. In contrast, the C-termini vary in length and show no apparent sequence homology, except for the region nearest to transmembrane domain-2 (North, 2002). The functional channels are composed of at least three subunits, which can form ion permeable pores through homo- and heteropolymerization (Nicke et al., 1998, 2005). P2XR subtypes differ with respect to their ligand-selectivity profiles, antagonist sensitivity, and cation selectivity. Their activation leads to an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$), with Ca^{2+} influx occurring through the pores of these channels and voltage-gated Ca^{2+} channels, following the initial depolarization of cells by P2XR-generated Na^+ currents (Koshimizu et al., 2000a; Samways and Egan, 2007). These receptor channels are expressed in numerous tissues, including the central nervous system, sensory, motor and sympathetic neurons, chromaffin cells, smooth muscle, testis, colon, gut, bladder, thymus, pancreas, skin, and immune cells (Burnstock, 2007a).

ATP, UTP, ADP, and UDP are native agonists for the P2YRs. To date, eight mammalian P2YRs have been identified and are denoted as $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, $P2Y_{11}R$, $P2Y_{12}R$, $P2Y_{13}R$, and $P2Y_{14}R$ (Fischer and Krugel, 2007). The missing numbers represent either non-mammalian versions of these receptors or proteins that have some sequence homology to P2YRs but do not respond to nucleotide application. In contrast to P2XRs and ARs, the genes of all P2YRs, other than $P2Y_{11}R$, do not contain introns in their coding sequence (Burnstock, 2007a). Four receptors, $P2Y_1R$, $P2Y_2R$, $P2Y_4R$, and $P2Y_6R$, signal through the $G_{q/11}$-dependent pathway, leading to the activation of phospholipase C and the generation of $InsP_3$ and diacylglycerol. In excitable cells, $InsP_3$-induced Ca^{2+} mobilization is frequently accompanied with an initial hyperpolarization of the cell membrane and an inhibition of voltage-gated Ca^{2+} influx mediated by Ca^{2+}-controlled K^+ channels. This is subsequently followed by a sustained depolarization of the plasma membrane and the facilitation of voltage-gated Ca^{2+} influx through poorly characterized pathways. Activation of MAP kinase and phospholipase D signaling pathways, both secondary to the activation of protein kinase C, has also been reported for P2YRs, as well as their coupling to $G_{i/o}$ ($P2Y_{13}R$) and G_s ($P2Y_{11}R$) signaling pathways (Erb et al., 2006). These receptors are expressed in the brain, lymph nodes, bone marrow, placenta, stomach, intestine, adipose tissue, epithelial, endothelial, and immune cells, osteoclasts and osteoblasts, platelets and other cell types (Burnstock, 2007a).

Adenosine is a common agonist for four subtypes that comprise the AR family of G protein-coupled receptors, designated A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. Each of these receptor genes contains an intron within the coding region, and polymorphisms have been observed in the A_1R and $A_{2A}R$. Activation of these receptors leads to modulation of basal adenylyl cyclase activity. The A_1R and A_3R are negatively coupled to the adenylyl cyclase signaling pathway through pertussis toxin-sensitive $G_{i/o}$. The $A_{2A}R$ and $A_{2B}R$, on the other hand, stimulate the activity of this enzyme through cholera toxin-sensitive G_s. The $A_{2B}R$ also signals through $G_{q/11}$-dependent phospholipase C. The intracellular pathways triggered by these receptors include modulation of the activity of voltage-gated Ca^{2+} channels and K^+ channels, as well as activation of the MAP kinase family of signal transduction pathways. The physiological relevance of these receptors in modulation of cardiovascular, immune and central nervous system have been confirmed by transgenic knockout mice (Ralevic and Burnstock, 1998; Burnstock, 2007a).

All elements required for purinergic signaling are present in the pituitary gland. ATP is released by magnocellular neurons in the posterior pituitary and by anterior pituitary cells. The pituitary gland also contains nucleotide and nucleoside-converting enzymes whose activities account for the formation of ADP and adenosine. The cells of this gland express numerous ARs, P2YRs, and P2XRs. In this paper, we will review the current state of knowledge regarding purinergic signaling in the magocellular neurons and the pituitary gland. Earlier work in this field has been summarized in several reviews (Stojilkovic et al., 2000; Stojilkovic and Koshimizu, 2001; Troadec and Thirion, 2002; Rees et al., 2003a,b). For the role of purinergic signaling in parvocellular hypothalamic nuclei see (Burnstock, 2007b).

2. Release and extracellular metabolism of ATP

The physiological sources of the extracellular nucleotides required for activation of purinergic receptors in pituitary cells remain largely uncharacterized. In general, neurons, neuroendocrine cells and platelets release ATP by Ca^{2+}-controlled exocytosis of nucleotides stored within synaptic vesicles or dense core granules. The magnocellular neurons of the hypothalamus that control release of vasopressin and oxytocin also contain ATP and the specific pattern of action potentials originating from the cellular bodies of these neurons has been suggested to control the release of ATP (Troadec and Thirion, 2002). However, inhibition of electrical activity in the magnocellular neurons only partially inhibits secretion, suggesting that afferent signals acting on nerve terminals in the neurohypophysis may also contribute to ATP release (Sperlagh et al., 1999). It has also been suggested that extracellular ATP levels could reach 4–40 μM, which are sufficient concentrations to activate the majority of P2Rs (Troadec et al., 1998). A more recent study confirmed that ATP released from the neurohypophysis during stimulation depolarizes nerve terminals and potentiates vasopressin secretion (Knott et al., 2008). In addition to its action on nerve terminals in the posterior pituitary, released ATP probably acts on neurohypophysial astrocytes, also known as pituicytes, but does not affect oxytocin release (Troadec et al., 1998; Knott et al., 2008).

We recently showed that ATP is also released by normal and immortalized anterior pituitary cells at resting conditions. Such basal ATP release was enhanced in cells treated with ARL67156, an inhibitor of ectonucleotidases (He et al., 2005). 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 (Tomic et al., 1996). This is consistent with an earlier study showing calcium-dependence of ATP release (Chen et al., 1995) and modulation of ATP release by prolactin secretagogues (Nunez et al., 1997). On the other hand, facilitation of prolactin release did not elevate ATP secretion in perifused pituitary cells, suggesting that it could also be released by another mechanism, possibly from pituicytes. Consistent with our findings, both secretory and non-secretory ATP release pathways were found to

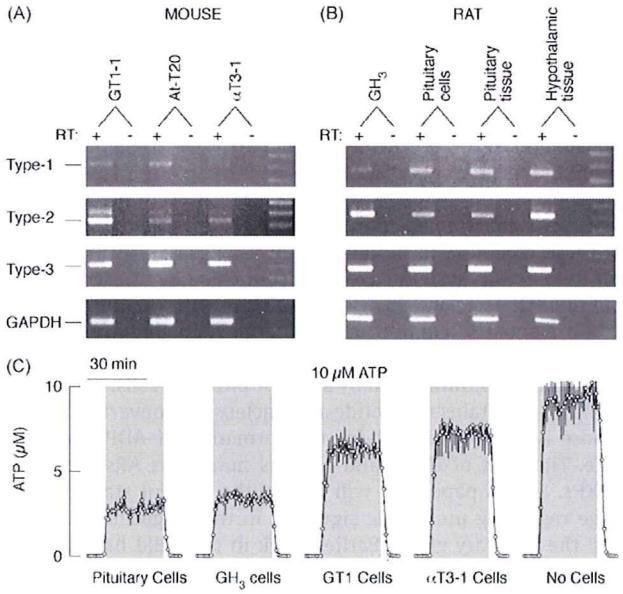

Fig. 1. Expression and activity of ectonucleotidase in hypothalamic and pituitary tissues, pituitary cells, and immortalized hypiothalamic (GT1-1) and pituitary (At-T20, αT3-1, and GH_3) cells. (A and B) Expression of ectonucleotidase eNTPDase 1–3 transcripts in hypothalamic and pituitary tissues and cells. In both panels, DNA markers are shown in the last right lanes. In this and following figures, for negative controls, PCR was conducted performing first-stand cDNA synthesis without RT (−). (C) Cell-type specificity of extracellular ATP metabolism. Chambers without and with cells (5×10^6 per column) were perifused with Krebs–Ringer buffer at flow rate of 0.8 ml/min. Samples were collected every minute and immediately tested for ATP concentrations. *Gray areas* indicate the duration of ATP application and its concentrations in the medium. *Traces* show measured ATP concentrations in effluents.

be operative in human astrocytes (Joseph et al., 2003). In other tissues, ABC-binding cassette transporters, hemichannels, and P2X7R, among others have been suggested to participate in non-vesicular ATP release (Abbracchio et al., 2009). Interestingly, pituitary cells express functional multidrug resistance proteins (Andric et al., 2006) and P2X7R (Koshimizu et al., 2000b), which could contribute to ATP release. Finally, we showed that endogenous release of ATP by cultured cells is of sufficient amplitude to desensitize homomeric and heteromeric P2X3Rs, confirming that the measured ATP is biologically active (He et al., 2005). Certainly, these *in vitro* conditions should not accurately reflect the *in vivo* situation, where the tissue ATP concentration represents a balance between rates of release, hydrolysis, and dilution into intercellular compartments.

The action of ATP as an autocrine/paracrine factor is critically dependent on its rapid metabolism by ectonucleotidases (Zimmermann, 2000). These enzymes are also expressed on the plasma membrane of pituicytes and neurosecretory terminals (Thirion et al., 1996). Extracellularly added ATP is rapidly hydrolyzed by isolated neurohypophysis, indicating that the ectonucleotidases are functional. The group that discovered this also detected accumulation of adenosine when AMP was added to isolated neurohypophysis, suggesting that these enzymes provide a pathway for the activation of ARs in this tissue. This, in turn, is likely to account for the termination of ATP-induced vasopressin release (Sperlagh et al., 1999). mRNA transcripts for plasma membrane-located eNTPDases 1, 2 and 3 are expressed in hypothalamic and pituitary tissues, cultured pituitary cells and immortalized lacto-somatotrophs, corticotrophs, gonadotrophs, as well as in GnRH-secreting cells (Fig. 1A and B) (He et al., 2005). Depending on the cell type and the rates of perifusion, pituitary cells were able to degrade between 30 and 70% of extracellularly added ATP (Fig. 1C). The ectonucleotide cascade not only terminates the extracellular messenger functions of ATP but also provides a pathway for the generation of ADP and adenosine, which in pituitary cells may activate some P2YRs and ARs, respectively (see below). Ecto-5′-nucleotidases (CD73), which generate adenosine from AMP, were found by immunocytochemistry in about 20% of anterior pituitary cells (Lewis et al., 2006).

3. Expression and signaling by P2XRs

Initial knowledge about the expression and role of P2Rs in the posterior pituitary was obtained in experiments using Ca^{2+} measurements and hormone secretion. These studies revealed that extracellularly added ATP lead to an increase in $[Ca^{2+}]_i$ and vasopressin secretion, and that these actions were strongly and reversibly inhibited by the P2R blocker suramin (reviewed in (Troadec and Thirion, 2002)). P2XRs have been suggested to mediate the action of extracellular ATP on vasopressin release (Kapoor and Sladek, 2000). Initially, the role of P2X2Rs in ATP-induced increases in $[Ca^{2+}]_i$ and peptide release from rat isolated neurohypophysial terminals had been suggested (Troadec et al., 1998). A more recent study indicated that a functional mixture of homomeric P2X2R and P2X3R mediates the majority of ATP's responses in vasopressinergic neurohypophysial terminals. The same study also provided the first electrophysiological evidence for the existence of P2XR currents in vasopressinergic neurohypophysial terminals and showed the lack of expression of P2XRs in terminals labeled for oxytocin (Knott et al., 2005). ATP also plays a role in vasopressin release by activating postsynaptic receptors on perikarya or dendrites of supraoptic neurons (Song and Sladek, 2006). At the present time, there is no evidence for the functional role of P2XRs in pituicytes.

Single-cell $[Ca^{2+}]_i$ measurements, along with studies on hormone secretion in cells stimulated with various agonists in the presence and absence of P2XR inhibitors were also instrumental in the initial characterization of purinergic receptors in anterior pituitary cells (Tomic et al., 1996; Nunez et al., 1997; Koshimizu et al., 2000b; Villalobos et al., 1997; Chung et al., 2000). These experiments revealed that functional P2XRs are operative in all secretory cell types and raised the possibility that several subtypes of these channels could be expressed in a cell type-specific manner. However, this method is of limited use for the identification of the receptor subtypes expressed, especially in characterizing the rapidly desensitizing homomeric and heteromeric P2XRs, and their roles in electrical activity (He et al., 2003a).

In more recent studies, we combined molecular biology techniques with electrophysiology to obtain additional data on expression and roles of these channels in pituitary cells. With the use of specific rat P2X primers and sequence analysis, P2X2, P2X3, P2X4, and P2X7 mRNA transcripts were detected in a mixed population of anterior pituitary cells (Fig. 2A, lane 1). Immortalized GH_3 pituitary cells also expressed transcripts for P2X3, P2X4, and P2X7 subunits, but not the P2X2 subunit (Fig. 2A, lane 4). The mRNA levels of P2XRs in anterior pituitary cells were also examined by *in situ* hybridization. Fig. 2A, top left panel, shows the position of the anterior and posterior pituitary. In parallel to RT-PCR analysis, the mRNA hybrids of the P2X2, P2X3, P2X4, and P2X7 subunits were also present in the anterior pituitary. The P2X4 mRNA subunit was more pronounced in the anterior than in the posterior pituitary, whereas the expression of the P2X3 subunit was relatively low in both areas. These mRNA expression patterns were consistent with immunohistochemical studies in the rat hypothalamo-neurohypophysial system at the electron microscope level. Immunoreactivity to P2X2Rs was localized in paraventricular and supraoptic nuclei, as well as in the posterior pituitary—in pituicytes and a subpopulation of neurosecretory axons (Loesch et

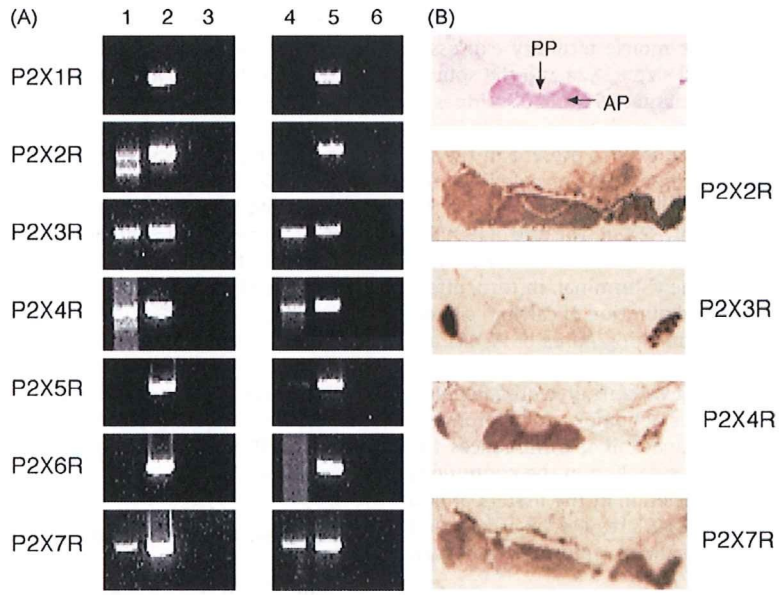

Fig. 2. Expression of P2XR mRNAs in the pituitary gland. (A) Detection of mRNA transcripts for P2XRs in anterior pituitary cells (line 1) and a GH_3 cell line (4). Lines 2 and 5 indicate positive controls and lines 3 and 6 negative controls. (B) Detection of mRNA transcripts in the rat pituitary by *in situ* hybridization. PP, posterior pituitary; AP, anterior pituitary.

al., 1999). Immunocytochemical labeling in vasopressinergic terminals also suggested the existence of P2X2, P2X3, P2X4, and P2X7 subunits (Knott et al., 2005).

Abundant PCR products of two different sizes, approximately 1.6 and 1.4 kb long, were consistently amplified by the P2X2 primers in mixed rat pituitary cells (Fig. 1A). Earlier experiments revealed that the primary P2X2 gene transcript in rats undergoes extensive alternative splicing resulting in modified mRNA sequences. One of the spliced subunits, termed P2X2b, lacks a series of 69 C-terminal amino acids and creates a functional homomeric channel, which desensitizes more rapidly than the full-size receptor, termed P2X2a (Koshimizu et al., 1999, 1998). This form of the subunit was also detected in other tissues (Brandle et al., 1997; Simon et al., 1997; Parker et al., 1998). It has also been suggested that electrostatic charges of six amino acid side chains located close to the proximal splicing site play a critical role in controlling the rate of receptor desensitization (Koshimizu et al., 1999).

Recently, we also analyzed the expression of P2X2Rs in mouse pituitary cells and investigated the functional significance of the N- and C-termini specific structures of these receptors in terms

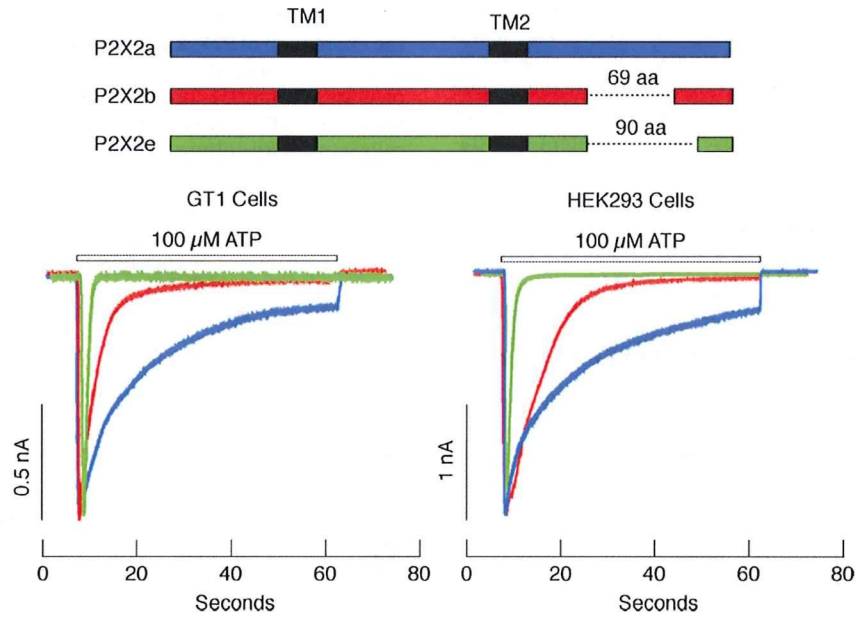

Fig. 3. Electrophysiological characterization of P2X2Rs cloned from mouse anterior pituitary cells. *Top panel*, schematic representation of splice forms of P2X2R in pituitary cells. Horizontal dotted lines indicate missing sequences. Black areas indicate transmembrane domains (TM1 and TM2). *Bottom panels*, patterns of ATP-induced current profiles in GT1 neurons (*left*) and HEK293 cells (*right*) expressing recombinant P2X2Rs. Green traces, P2X2e currents; red traces, P2X2bR currents; blue traces, P2X2aR current. Horizontal bars indicate duration of ATP application. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of current, calcium signaling, and inter-subunit interactions in living cells. This study showed that the mouse pituitary expresses three forms of P2X2 subunits: the full size P2X2a and the spliced forms P2X2b and P2X2e, which are missing 69 and 90 residues in their C-termini, respectively. Furthermore, all three mouse forms have an additional 13 N-terminal residues not present in the rat pituitary gland. Electrophysiological experiments revealed that the rate of homomeric P2X2eR desensitization was comparable to the rates of rapidly desensitizing P2X1R and P2X3R. The rate of P2X2bR desensitization, however, was faster than P2X2aR but slower than P2X2eR (Fig. 3). These deletions in the C-terminal, in turn, effectively reduced the peak amplitude and duration of calcium signals (Koshimizu et al., 2006). The ion-permeating pores of P2XRs are formed by three subunits, and because the same primary P2X2 transcript can yield three different splicing variants, it is probable that a single pituitary cell expresses a series of homo- and heteromeric P2X2Rs. The structure of the N-terminal tail also influences the duration of P2X2R-mediated channel signaling in the continuous presence of an agonist. Specifically, deletion of the first 13 amino acids at the N-terminus of the mouse P2X2R resulted in accelerated desensitization rates of all C-terminal splicing isoforms (Koshimizu et al., 2006).

Single-cell patch-clamp analysis in gonadotrophs from embryonic, neonatal, and adult rats revealed that ATP application in all gonadotrophs generates a depolarizing and non-oscillatory current (Fig. 4B), in contrast to GnRH that triggered an oscillatory hyperpolarizing current (Fig. 4A). This clearly indicates that Ca^{2+}-mobilizing P2YRs are not expressed in this particular cell type. The biophysical and pharmacological properties of ATP-induced depolarizing current (kinetics of activation, deactivation, desensitization, and resensitization) in gonadotrophs from adult and neonatal animals were comparable with those observed in cells expressing recombinant P2X2R. Sensitivity of ATP-evoked current to inhibition by pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulfonic acid, reactive blue 2, and suramin, as well as the lack of effect of ivermectin, further confirmed this conclusion. ATP-induced rapid depolarization of gonadotrophs leads to the initiation of firing in quiescent cells, an increase in the frequency of action potentials in spontaneously active cells (Fig. 4C), and a transient stimulation of LH release. ATP also influenced GnRH-induced currents and membrane potential oscillations as well as LH release in an extracellular Ca^{2+}-dependent manner. These $InsP_3$-dependent oscillations were facilitated, slowed, or stopped, depending on the ATP concentration, the time of its application, and the level of Ca^{2+} content in intracellular stores. These results indicate that in gonadotrophs P2X2Rs could operate as pacemaking channels and modulators of GnRH-controlled electrical activity and secretion (Zemkova et al., 2006).

Approximately 90% of identified lactotrophs, from neonatal to adult, also responded to ATP application by generating inward depolarizing currents. Although the average peak amplitude of currents in response to supramaximal ATP concentrations was several-fold higher in gonadotrophs than in lactotrophs, the profiles of currents were highly comparable in the two cells types. Ivermectin, the specific P2X4R allosteric modulator, affected the ATP-induced current in lactotrophs but not in gonadotrophs. In the presence of ivermectin, there was large, roughly 6-fold increase in the maximum amplitude of current. Ivermectin also significantly increased the sensitivity of channels for ATP, delayed the deactivation of receptors, and enhanced ATP-induced PRL release. These results indicate that lactotrophs express homomeric and/or heteromeric P2X4Rs, which could operate as pacemaking channels (Varva et al., 2008).

4. Expression and signaling by P2YRs

Since pituicytes are intimately associated with neurosecretory terminals, the released ATP from neuronal endings could affect their function in neurohypophysis. In the majority of pituicytes in primary culture, ATP triggers a rapid extracellular calcium-independent rise in $[Ca^{2+}]_i$, which is abolished in cells when phospholipase C and the endoplasmic reticulum calcium pump are blocked, indicating the functional operation of calcium-mobilizing P2YRs (Troadec et al., 1999). The stimulatory action of ATP is followed by potassium efflux (Troadec et al., 2000). The identification of the P2YR mediating the action of ATP in pituicytes has not been completed. The pharmacological profile of the calcium response in these cells suggests that ADP, UTP, and UDP are not endogenous agonists for these receptors (Troadec et al., 1999).

Sheep and rat anterior pituitary cells also express P2YRs and their activation by ATP is associated with an elevation in $[Ca^{2+}]_i$ due to $InsP_3$-dependent release of Ca^{2+} from intracellular stores (van der Merwe et al., 1989; Davidson et al., 1990). Molecular cloning and the functional characterization of rat P2YRs in the pituitary revealed the expression of $P2Y_2R$ with a pharmacological profile resembling that of the sheep pituitary (Chen et al., 1996a). For this type of receptor, ATP and UTP are equipotent agonists. The presence of UTP-sensitive P2YRs has been suggested in rat pituitary folliculo-stellate cells in primary culture (Uchiyama et al., 2001). The expression of

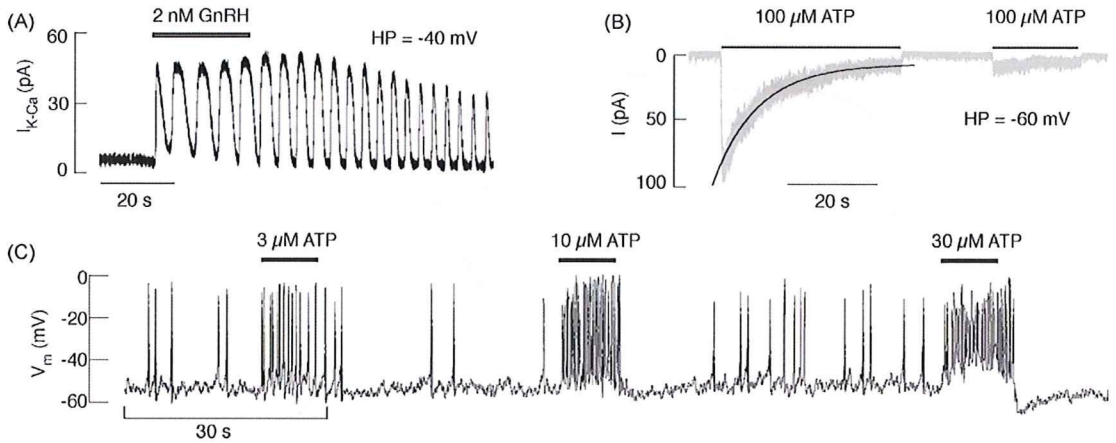

Fig. 4. ATP-induced current and pacemaking activity in identified rat pituitary gonadotrophs. (A) GnRH-induced outward oscillatory calcium-activated SK type potassium current. HP, holding potential. (B) ATP-induced non-oscillatory inward current. *Gray trace*, experimental record; *black solid line*, the monoexponential fitting curve for the decay of current in the presence of ATP (desensitization). (C) Concentration-dependent effects of ATP on the frequency of action potentials and the level and duration of afterhyperpolarization in a spontaneously active cell. Horizontal bars indicate duration of agonist application.

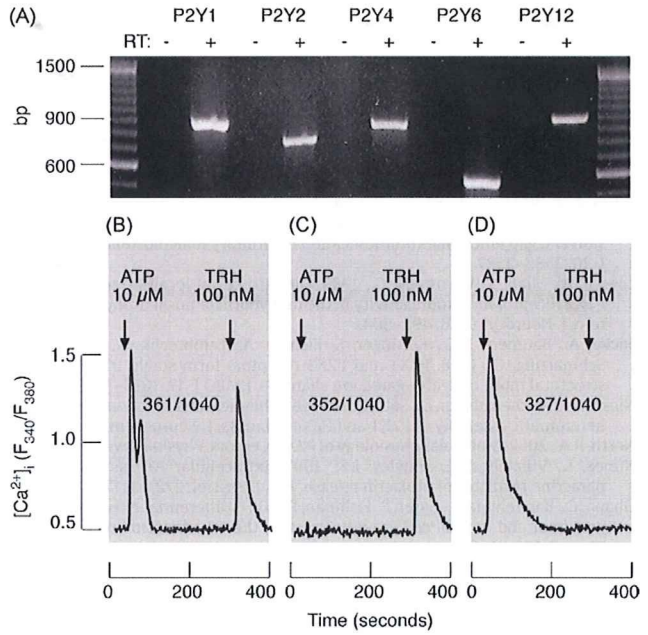

Fig. 5. Characterization of P2YRs expressed in rat anterior pituitary cells. (A) Detection of P2YR mRNA transcripts in a mixed population of anterior pituitary cells. (B–D) ATP (10 μM)-induced calcium mobilization from endoplasmic reticulum in pituitary cells bathed in calcium-deficient medium. Numerators indicate number of cells responding to ATP and TRH ((B) lactotrophs), TRH only ((C) lactotrophs), and ATP only ((D) unidentified pituitary cells). Arrows indicate moments of agonist applications, which were present in medium to the end of recordings.

these receptors has also been suggested in gonadotrophs (Chen et al., 1996b) and lactotrophs (Carew et al., 1994), but our laboratory was unable to confirm these findings (Tomic et al., 1996; He et al., 2003b).

In addition to $P2Y_2R$, RT-PCR analysis revealed the presence of transcripts for G_q-coupled calcium-mobilizing $P2Y_1R$, $P2Y_4R$, and $P2Y_6R$, as well as G_i-coupled $P2Y_{12}R$, in mixed anterior pituitary cells (Fig. 5A). Unlike UTP and UDP, in the absence of extracellular Ca^{2+}, ATP and ADP triggered calcium signaling in about 40% of lactotrophs and prolactin release in mixed populations of secretory pituitary cells. A fraction of other unidentified cells also responded to ATP/ADP application with a rise in $[Ca^{2+}]_i$ (Fig. 5B–D). The ligand-selectivity profile of calcium mobilization-dependent signaling and the blockade of these responses by pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulfonic acid indicated that $P2Y_1Rs$ could be responsible for extracellular Ca^{2+}-independent stimulation of prolactin release, but further pharmacological characterization of these receptors is required (He et al., 2003b).

5. Expression and signaling by ARs

Extracellular ATP has a profound effect on the three-dimensional structure of pituicytes; it changes them from a flat shape to a stellate morphology. However, this action of ATP is not mediated by P2Rs, but rather requires both its hydrolysis by ectonucleotidases and the activation of ARs (Rosso et al., 2002). The action of adenosine in the posterior pituitary is not restricted to astroglial cells, but also activates ARs expressed in neurohypophysial terminals, leading to the inhibition of N-type calcium channels. In turn, this attenuates high K^+-induced release of vasopressin and oxytocin by activating the A_1R subtype (Wang et al., 2002). Conversion of ATP to adenosine also terminates the stimulatory action of endogenously released and exogenously added ATP on vasopressin release from hypothalamo-neurohypophysial explants (Song and Sladek, 2005; Knott et al., 2007). These results indicate the dual control of the stimulatory action of ATP on neuropeptide release by ectonucleotidases and the subsequent activation of A_1Rs.

The expression and role of A_1Rs in the anterior pituitary gland has been reviewed (Rees et al., 2003a,b). Briefly, functional receptors in immortalized pituitary cells were first described in 1985 (Dorflinger and Schonbrunn, 1985). Later, they were also identified in pituitary lactotrophs (Yu et al., 1998; Scorziello et al., 1993). Pharmacological, electrophysiological, and secretory data indicated the negative coupling of these receptors to the adenylyl cyclase signaling pathway through pertussis toxin-sensitive G proteins, causing the abolition of spontaneous electrical activity, Ca^{2+} signaling, and hormone release. Cessation of spontaneous electrical activity by adenosine probably occurs indirectly, by the activation of voltage-gated K^+ channels, and/or directly by the inhibition of voltage-gated Ca^{2+} channels. Pituitary cells also express $A_{2A}R$, $A_{2B}R$, and A_3R (Weaver, 1993; Dixon et al., 1996; Ohana et al., 2001). The $A_{2B}Rs$ are expressed in folliculo-stellate cells, stimulate IL-6 secretion (Rees et al., 2003c), and trigger the formation of gap junctions (Lewis et al., 2006). A_3Rs could contribute to the antiproliferative activity of adenosine in pituitary tumor cells (Ohana et al., 2001).

6. Concluding remarks

Once secreted by pituitary cells, the extracellular messenger actions of ATP are controlled by ecto-ATPases and ecto-5′-nucleotidase, which degrade ATP to ADP, AMP, and adenosine. While the capacity of these enzymes to hydrolyze ATP and generate adenosine from extracellular AMP is well established, further work is required to clarify the mechanism of ATP release by posterior and anterior pituitary cells. ATP, ADP, and adenosine are native agonists for pituitary cells and have dual actions on Ca^{2+} signaling: stimulatory and inhibitory, depending on the receptor subtype expressed. ATP activates P2XRs, which are expressed in all secretory anterior pituitary cells, as well as in nerve terminals in the posterior pituitary. There is molecular evidence for the expression of P2X2, P2X3, P2X4, and P2X7 subunits and immunocytochemical evidence for the expression of the P2X6 subunit. Electrophysiological studies have established the expression of P2X2Rs in vasopressinergic neurohypophysial terminals and gonadotrophs, as well as the expression of P2X4R in lactotrophs. These receptors facilitate the firing of action potentials, elevate $[Ca^{2+}]_i$, and stimulate hormone release. The identification of the P2XR subtype(s) expressed in somatotrophs, thyrotrophs, corticotrophs, and pituicytes necessitates further investigation. Molecular and physiological evidence for the expression of several Ca^{2+}-mobilizing P2YRs has also been obtained. In contrast to P2XRs, the expression of these receptors is more limited in the anterior pituitary gland. A fraction of lactotrophs probably expresses $P2Y_1R$, whereas folliculo-stellate cells express $P2Y_2Rs$. Other secretory anterior pituitary cells also express P2YRs, but their identification will require additional studies. ADP is a potent agonist for the activation of $P2Y_1R$ in anterior pituitary cells. Posterior and anterior pituitary cells also express ARs. Among the members of this family of receptors, the best characterized are the A_1Rs. These receptors are coupled to the $G_{i/o}$ signaling pathway and effectively attenuate electrical activity through multiple mechanisms. Such organization of the purinergic pathway in the pituitary gland provides an effective but transient mechanism for the action of ATP as an agonist. The details of this mechanism include the prolonged activation of $P2Y_1R$ by ADP as well as termination of the stimulatory actions of P2XRs not only by hydrolysis of ATP, but also by the generation of adenosine and activation of A_1Rs. Future work on the mechanism of purinergic release and the distribution of specific purinergic signaling elements should help us unravel the spatial and temporal aspects of ATP's actions in the pituitary gland.

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Forum Minireview

New Topics in Vasopressin Receptors and Approach to Novel Drugs: Vasopressin and Pain Perception

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Abstract. Arginine vasopressin (AVP) activates three vasopressin receptors and it also has an agonistic activity on the oxytocin receptor. For an accurate description of the target receptor subtype(s) responsible for complex AVP and oxytocin actions, a careful evaluation of ligand specificity and receptor activities are required, particularly when these receptors are co-expressed in the central nervous system. Previous studies suggest that AVP plays a regulatory role in nociception through the direct activation of central vasopressin receptors and also through the receptors that reside in the peripheral tissues. Genetically altered rodent models, including the AVP-deficient mutant Brattleboro rat and gene knockout mice lacking an endogenous opioid peptide, advanced the understanding of the interactions between the pain perception process and AVP system. This report reviews previous findings in this important field and reconciles them with the findings of recent gene knockout/knockdown studies.

Keywords: arginine vasopressin, oxytocin, pain, nociception, V1a receptor, V1b receptor

Introduction

Neurohypophyseal hormones, such as arginine vasopressin (AVP) and oxytocin (OT), have a wide range of effects on the central nervous system (CNS), including nociception, learning and memory process, social recognition, central regulation of the cardiovascular system, and stress response (1 – 13). These CNS functions of AVP are not directly related to their peripheral roles as circulating hormones (14). Indeed, neurons containing AVP peptides extend to extrahypothalamic structures, which are important for pain perception (15 – 19). OT, on the other hand, is present in several thalamic nuclei, the mesencephalic central gray nucleus, the substantia nigra, the locus coeruleus, the raphe nucleus, the nucleus of the solitary tract, and the spinal cord (20, 21).

Because AVP is a principal agonist to all vasopressin receptors and also has agonistic activity on OT receptors, it has been difficult to define the target receptor responsible for the complex AVP actions in the CNS. There are three types of G-protein–coupled vasopressin receptors, termed V1a, V1b, and V2 receptors (22); and a single OT-receptor gene has been identified (20). V1a, V1b, and OT receptors activate Gq heterotrimeric GTP binding protein and V2 stimulates Gs protein (22). The main receptors for AVP in the brain could be presumably of the V1 type (23); no V2-specific ligand binding or V2 mRNA were detected in previous reports (24, 25), while other studies suggest that there is a V2-like receptor in the CNS (3, 26). A relatively good correlation was reported between the autoradiographic distribution of mRNA transcripts for the V1a and autoradiographic distribution reported for V1-specific binding sites (25, 27). The V1b receptor transcript, on the other hand, is localized in several brain regions, including the olfactory bulb, CA2 pyramidal neurons in the hippocampus, supraoptic, suprachiasmatic and dorsomedial hypothalamic nuclei, piriform and entorhinal cortices, substantia nigra, and dorsal motor nucleus of the vagus (28 – 30). The functional interactions between AVP /OT peptides and their receptor system have been shown to provide diverse opportunities to modulate the efficiency of sensory transmission (31). This review will

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discuss the roles of endogenous and external AVP in nociception. Owing to limitations of space, this article is not a comprehensive overview of the subject. For discussions concerning the variety of neurophysiological roles of central AVP and OT, other previous reviews should also be consulted (3, 14, 20, 21, 32 – 35).

Effect of AVP in pain perception

The pain perception process can be altered by a variety of peptide and non-peptide neurotransmitters (33). Using rodent withdrawal reflex tests, such as the tail flick test, or more complex behavioral tests, such as the hot plate test, in which supraspinal motivational processing is involved, AVP and OT were shown to modify the nociception threshold induced by these noxious heat stimuli (33). In early reports, the antinociceptive activity was observed after either the intraventricular or subcutaneous administration of lysine vasopressin (LVP) in the rat or after intraperitoneal injection of AVP in mice (1, 36, 37). Des-glycinamide-LVP, a vasopressin analog with no apparent pressor or antidiuretic action, or des-amino-AVP, a vasopressin analog with minimal pressor activity but greatly enhanced antidiuretic activity, was also relatively ineffective (36, 37). Later, intracerebroventricular injections of AVP or OT were shown to lead to antinociceptive effects in rats and in human cancer patients (38 – 40).

In addition to the capacity of administered AVP to show antinociception, studies on nociceptive condition of Brattleboro rats, which are deficient in mature vasopressin, are informative concerning the role of AVP in pain sensitivity. When examined using the flinch-jump threshold test, Brattleboro rats were in a hyperalgesic state and stress analgesia was impaired in comparison to controls (41). These analgesic deficits observed in Brattleboro rats was vasopressin-dependent; LVP introduced into the lateral cerebral ventricle, or subcutaneously at a high dose, induced an antinociceptive effect (42). One of other methods to inhibit AVP action in vivo is the administration of antiserum against AVP directly into the cerebral ventricle or brain nucleus. Administration of antiserum to the cerebral ventricle resulted in a small reduction of tail-flick latency from 3.9 s in the vehicle adminstration to 3.3 s at a high radiant heat level (50°C), but it prolonged the latency at moderate (46°C) heat (43).

In the CNS, AVP is released synaptically in the lateral septum, hippocampus, amygdala, habenula, and several other brain structures (15). Therefore, the brain region(s) where AVP acts as antinociceptive neurotransmitter is of large concern. The amygdala, an important region regulating emotional responses, such as anxiety or fear, has a critical role in conditional pain perception. When AVP was injected into the central nucleus of the amygdala and the nociceptive jaw opening reflex monitored in freely moving rats, AVP showed an analgesic effect in diagnostic electromyograms, which was inhibited by a V1, but not V2, receptor antagonist (44). Other studies examined the effect of AVP in nociception by injecting it into the periaqueductal gray and observed an increase in pain threshold (26). AVP increased the endorphin and enkephalin concentration in liquid samples perfused through the periaqueductal gray (45). Not only intracranial injection, but also intrathecal administration of AVP showed an antinociceptive effect (46, 47). However, another study reported that AVP failed to influence nociceptive thresholds or to modify the antinociceptive action of morphine (48). Intrathecal AVP also produced scratching bouts and suppression of hindbody motor function (47).

Opioid-dependency of AVP-induced antinociception

So far, evidence has been accumulating that AVP administered into the intracranial and, in some reports, intrathecal spaces causes an increase in the pain threshold. The next question to be answered is whether the pain-inhibiting effect of AVP could be mediated by intrinsic opioid peptide–receptor systems. To clarify this, a selective μ-opioid–receptor antagonist, naloxone, or several opioid agonists, such as morphine and other peptide agonists, were used together with AVP. The series of results so far have been equivocal. Naloxone inhibited AVP-induced analgesia in several studies (44, 49, 50), but in other studies, the antinociceptive actions of AVP were not mediated by opioids (1, 37, 38, 46).

Apart from the intracranial regions, anterior and posterior pituitary functions are closely related to AVP and OT. Since AVP is secreted under stress conditions and several reports describe evidence of AVP-secreting stimulation also causing secretion of opioid peptides. In the posterior pituitary where AVP and OT neurons terminate, the hypophysial nerve terminals contain enkephalin peptides together with AVP or OT in rat pituitary (51). Dynorphin immunoreactivity was also localized in AVP and OT neurons (52). These reports suggested that AVP and opioid peptides could be secreted from the posterior pituitary and in the CNS. On the other hand, AVP acts as stimulator of adrenocorticotropin secretion in the anterior pituitary. The adrenocorticotropin peptide is processed from the pro-opiomelanocortin (POMC) gene. Although the POMC gene can also produce β-endorphin in the CNS of the human and rat pituitary, a lack of an appreciable amount of active β-endorphin has been reported in basal condi-

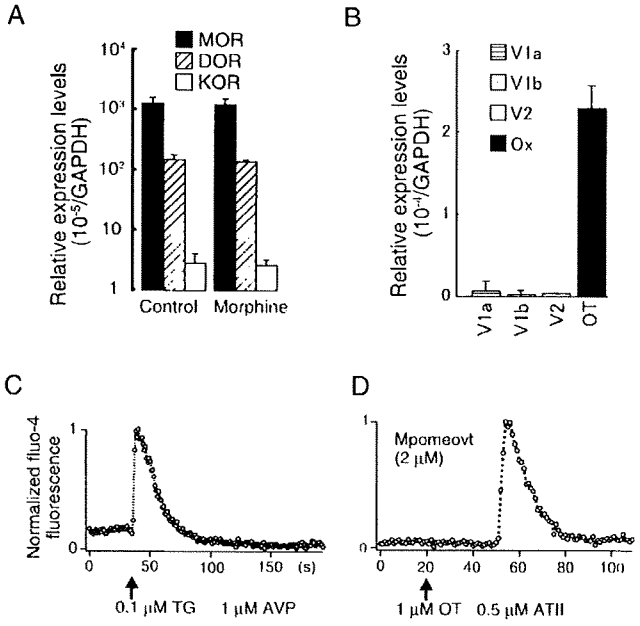

Fig. 1. The co-expression of MOR1 and DOR, but not κ (KOR), opioid receptors and OT oxytocin receptor transcripts in a human neuroblastoma cell line. Real time PCR analysis detected MOR1 and DOR (A) and OT receptor (B) transcripts as the predominant opioid receptors and vasopressin/OT receptor, respectively, in human neuroblastoma cells. C: The specific OT-receptor agonist TG stimulated $[Ca^{2+}]_i$ mobilization and desensitized the corresponding receptor, resulting in no Ca^{2+} response upon subsequent AVP (1 μM) application. The TG-induced $[Ca^{2+}]_i$ mobilization was inhibited by pretreatment of the cells with 2 μM Mpomeovt, a selective OT-receptor antagonist (D), but Mpomeovt failed to inhibit the angiotensin II–stimulated Ca^{2+} response. Mpomeovt: 1-deamino-2-*O*-methyl-tyrosyl-8-ornithine-1-(β-mercapto-(β,β-cyclopentamethylene)propionic acid)oxytocin; TG, [Thr^4,Gly^7] oxytocin.

tions and under corticotropin-releasing stimuli, such as AVP administration or insulin-induced hypoglycemia (53). Therefore, in mice the pituitary gland is one of the major sources of peripherally circulating β-endorphin, but its role in analgesia remains uncertain (54). Previous studies have shown that hypophysectomy may in fact enhance most types of opioid analgesia (55). Furthermore, the lack of stress-induced analgesia documented in β-endorphin–knockout mice may be due to the loss of central β-endorphin rather than loss of pituitary-derived β-endorphin (54).

OT and analgesia

Physiological stimulations that induce a large increase in OT concentrations in the blood, such as parturition and vaginal dilation, are known to raise the pain threshold (21). The analgesic effect of this type of OT action is not a morphine-sensitive process and peripheral injections of OT have no analgesic effect (21). OT, however, attenuates the development of tolerance to the analgesic action of morphine (56). Lesions of the hypothalamic paraventricular nucleus deplete endogenous immunoreactive AVP and OT from the rat spinal cord, but fail to modify the nociceptive thresholds (48). Therefore, complex interactions are suggested between the central OT systems and opioid analgesia.

Using a human neuroblastoma cell line the relationship of opioid- and OT-dependent cellular signaling was recently examined in our laboratory to determine the molecular mechanism for the enhanced nociceptive effect produced by OT. The neuroblastoma cells express both the μ-opioid receptor (MOR1) and δ-opioid receptor (DOR), in addition to the OT receptor (Fig. 1). OT receptor–Gq coupling and Ca^{2+} responses are evoked by application of a specific OT-receptor agonist, which

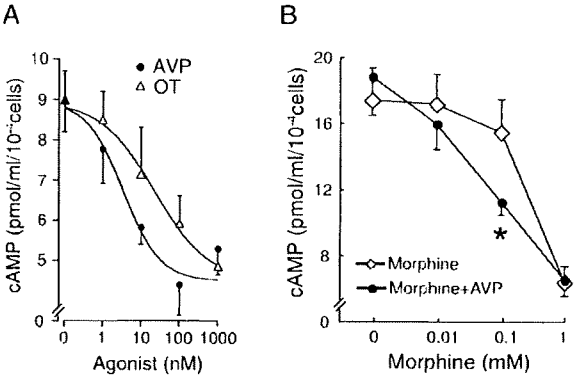

Fig. 2. The inhibition of cyclicAMP production by OT, AVP, and co-application of AVP and morphine. A: The inhibitory effects of OT and AVP against forskolin-induced cyclic AMP production. The cells were stimulated at the indicated concentrations of OT or AVP for 5 min at ambient temperature and reactions were terminated by heating cells at 100°C for 5 min. Cyclic AMP concentrations of the cellular extracts were examined by an enzyme-linked immunosorbent assay. B: The co-applications of morphine and AVP enhanced inhibition of adenylate cyclase activities. In the presence of 1 μM AVP, the inhibition of forskolin-stimulated adenylate cyclase by morphine was significantly enhanced. *P<0.05.

are inhibited by an antagonist, Mpomeovt (Fig. 1: C and D). Interestingly, OT inhibits forskolin-induced cyclic AMP production in a concentration-dependent manner (Fig. 2A). The co-application of 1 μM AVP and morphine enhances adenylate cyclase inhibition, when the effect is compared with morphine alone (Fig. 2B). These previously unpublished results from our investigations suggested that when expressed in the same cell and stimulated simultaneously, MOR1, DOR, and OT receptors cooperatively enhance the Gi-signaling pathway. These results might be one of the possible mechanisms for the analgesic effect of central OT-receptor activation.

Concluding remarks

A majority of the studies performed in vivo on the roles of central AVP and OT receptors suggested that these intrinsic peptides show analgesic effects through an undetermined mechanism. The complexities in delineating the AVP- and OT-receptor functions could partly originate from mutual receptor–ligand interactions. In addition, direct interactions between receptor molecules, resulting in homomer and heteromer receptor complexes, as well as indirect intracellular signaling cross talks, have also been suggested (57, 58). To obtain a more detailed picture, both genetically modified animal models and specific pharmacological tools continue to be useful by perturbing one signal domain so that the other remaining one could be more clearly demonstrated. In this regard, mice lacking the V1a- or V1b-receptor gene are important animal models for unequivocally identifying receptor subtype(s) responsible for AVP actions in the CNS [see the following review article in this issue by K Honda and Y Takano (ref. 59)].

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