NEW PERSPECTIVES ON ENDOCRINE SIGNALLING

Adenosine signalling pathways in the pituitary gland: one ligand, multiple receptors

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Abstract

Adenosine receptors are widely distributed in most species and mediate a diverse range of physiological and pathological effects. Although adenosine receptors have been identified in the pituitary gland, the distribution of the individual subtypes (A_1 , A_{2A} , A_{2B} , A_3) has not been well defined. Furthermore, the effects of adenosine on pituitary trophic activity and function are not well established despite good evidence for growth- and immune-modulating properties of the nucleoside elsewhere.

Recent advances have provided a more detailed description of adenosine receptor distribution and function in the anterior pituitary and this commentary reviews these observations and highlights some of the possible implications in relation to the control of the hypothalamic-pituitary-adrenal axis and the regulation of inflammation and pituitary cell growth.

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Introduction

Extracellular purines (adenosine, AMP, ADP and ATP) and pyrimidines (UDP and UTP) are important signalling molecules which mediate a diverse range of biological effects by activation of cell surface purine receptors. Purine receptors divide naturally into two families termed adenosine or P1 receptors, and P2 receptors, recognising mainly ADP, ATP, UDP and UTP. The cellular distribution and functions of P2 receptors in the pituitary gland have been studied extensively and reviewed elsewhere (Koshimizu *et al.* 2000, Stojilkovic & Koshimizu 2001). In contrast, until recently the location and biological effects of adenosine receptors in the pituitary have not been defined. Recent evidence, however, suggests that the distribution of these receptors may be cell-type specific and that their function within the pituitary is dependent upon this expression.

Classification of adenosine receptors

Adenosine receptors have been classified on the basis of convergent molecular, biochemical and pharmacological evidence into four subtypes, termed A_1 , A_{2A} , A_{2B} and A_3 receptors (Fredholm *et al.* 2001). All four receptor subtypes couple to G proteins and, in common with other G

protein-coupled receptors, possess seven transmembrane domains. They mediate a broad range of signalling responses, attributable in part to their G protein specificity (Fredholm *et al.* 2001), summarised in Table 1. The involvement of the different adenosine receptor subtypes is determined by using a combination of selective agonists and antagonists (Table 1). There are no compounds that are completely specific for any of the adenosine receptors. Putative action at the A_{2B} receptor is determined by the relative activity of 5'-N-ethylcarboxamidoadenosine (NECA) and 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680).

Distribution of adenosine receptors in the pituitary gland

Sattin and Rall (1970) used the ability of adenosine receptors to signal through cAMP to initially demonstrate their expression in the brain but it was not until the mid 1980s that their expression was shown in the anterior and later in the posterior pituitary when Anand-Srivastava and colleagues (Anand-Srivastava *et al.* 1985, Anand-Srivastava 1988) reported the presence of adenosine-sensitive adenylate cyclase in primary rat cultures of these tissues. These were shown to be A_2 receptors that also mediated

Table 1 Classification of adenosine receptors. Adenosine receptors activate a wide range of signal transduction pathways, in part related to their G protein specificity. A₁ receptors can activate several types of K⁺ channel (probably via β, γ-subunits) and couple to inhibition of Ca²⁺ currents (N-type, P-type, L-type) in a number of cell types. Stimulation or inhibition of the MAP kinase family of cascades may depend on the cellular context (Schulte & Fredholm 2000, Dubey *et al.* 2001). Selective agonists and antagonists for each receptor are shown, although all compounds lose their receptor subtype selectivity in high concentration. No selective agonists are currently available for the A_{2B} adenosine receptor

	A ₁	A _{2A}	A _{2B}	A ₃
G protein-coupling	G _{i/o}	G _s	G _s G _q	G _{i/o} G _q
Effects of G protein-coupling	↓ cAMP ↑ IP ₃ ↑ K ⁺ ↓ Ca ²⁺ currents ↑ MAP kinase	↑ cAMP ↑ MAP kinase	↑ cAMP ↑ IP ₃ ↑/↓ MAP kinase	↓ cAMP ↑ IP ₃ ↑ MAP kinase
Selective agonists	CCPA CPA	CGS 21680	None	IB-MECA 2Cl-IB-MECA
Selective antagonists	DPCPX	ZM241385	None	MRS1220

IP₃, inositol 1,4,5-triphosphate; CPA, N⁶-cyclopentyladenosine; CCPA, 2-chloro-CPA; CGS 21680, 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamido-adenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; IB-MECA, N⁶-(3-iodobenzyl)-5'-(N-methylcarbamoyl)-adenosine; 2Cl-IB-MECA, 2-chloro-IB-MECA; MRS1220, 9-chloro-2-(2-furyl)5-phenylacetylaminophenol; ZM241385, 4-(2-[7-amino-2-[2-furyl]]1,2,4)triazolo[2,3-a][1,3,5]triazin-5-ylamino)ethylphenol.

the release of adrenocorticotrophin (ACTH) (Anand-Srivastava *et al.* 1989). However, they failed to delineate which pituitary cell type expressed these receptors and it was not possible to determine which A₂ receptor subtype was present as the A_{2A} and A_{2B} subclasses had not been defined at this time.

Several investigators simultaneously examined the effects of adenosine on hormone release in rodent cell lines and hemipituitary sections. In rat GH₄C₁ cells, for example, adenosine inhibited growth hormone (GH) and prolactin (PRL) secretion (Dorflinger & Schonbrunn 1985). The authors also showed that R-PIA ((R)N⁶-phenylisopropyladenosine, a selective A₁ agonist) inhibited vasoactive intestinal peptide (VIP)-stimulated cAMP levels as well as the secretion of GH and PRL, indicating mediation via the A₁ adenosine receptor. This was the first description of functional A₁ adenosine receptors in pituitary cells. The expression of functional A₁ adenosine receptors in lactotrophs was later confirmed in GH₃ (Delahunty *et al.* 1988, Cooper *et al.* 1989, Mollard *et al.* 1991) and GH₄ (Navarro *et al.* 1997, Zapata *et al.* 1997) cells, and in primary lactotrophic cell cultures (Scorziello *et al.* 1993), with evidence of functional coupling to phospholipase C and calcium channels in addition to adenylate cyclase. The successful cloning of the rat A₁ adenosine receptor later enabled investigators to confirm A₁ receptor mRNA expression by Northern analysis in pituitary tissue (Reppert *et al.* 1991), although the cell-type distribution of the receptor was not examined.

Studies examining the mRNA expression of the A₂ receptor subtypes have also been undertaken in the pituitary gland. A_{2A} receptor mRNA expression has been reported to occur transiently during embryological devel-

opment of the rat anterior pituitary (Weaver 1993). A_{2B} receptor transcripts have also been detected in the pituitary gland (Stehle *et al.* 1992) but there are no reports of A₃ adenosine receptor expression although transcripts have been detected by RT-PCR in every other brain region studied in the rat (Dixon *et al.* 1996).

It is clear from these observations that A₁ and A₂ receptors are expressed in pituitary cells but the cell type(s) on which these receptors are located have not been defined. We attempted to address these limitations by analysing the distribution of A₁, A_{2A} and A_{2B} receptors in primary rat pituitary cell cultures and in rodent pituitary cell lines using a complementary range of methodologies (Rees *et al.* 2002). We were able to demonstrate the presence of functional A_{2B} adenosine receptors in primary anterior pituitary cell cultures and in pituitary folliculostellate cell lines by demonstrating a rank order of potency of NECA (universal adenosine receptor agonist) > adenosine > CGS 21680 (selective A_{2A} receptor agonist) on stimulating cAMP production. We were, however, unable to demonstrate such responses in pituitary endocrine GH₃ or AtT20 cells but confirmed the existence of functional A₁ receptors in these cells by demonstrating inhibition of VIP- or forskolin-stimulated cAMP production. These findings were supported by RT-PCR, immunocytochemistry and ligand binding studies. We thus showed that A_{2B} receptors are expressed mainly in folliculostellate cells and that A₁ receptors are expressed in both folliculostellate and endocrine cells. We also concluded that the A_{2B} receptor is functionally dominant in the anterior pituitary and that A₁ receptor expression in the cell lines is present in a low affinity conformation. These data highlighted the differential expression patterns of A₁ and A_{2B}

Figure 1 Distribution of adenosine receptors (A_1R , A_{2BR}) in pituitary endocrine and folliculostellate cells. A_1 adenosine receptors have been identified in GH_3 , GH_4 , GH_4C_1 and AtT20 pituitary endocrine tumour cell lines. Functional studies have identified coupling of these receptors to phospholipase C (PLC; possibly by cross-coupling of $G_{i/o}$ signalling pathway to PLC by $\beta\gamma$ dimer), Ca^{2+} channels (L-type) and adenylate cyclase (AC). Where studied, the A_1 receptor is found in a low affinity state in these cells. Recent studies have shown the A_{2B} receptor, positively coupled to adenylate cyclase, to be the predominant functional adenosine receptor expressed in folliculostellate cells, although mRNA and protein expression of the A_1 receptor subtype is also detectable. IP₃, inositol 1,4,5-triphosphate; PKC, protein kinase C; DAG, diacylglycerol; ERK, extracellular signal-related protein kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun amino terminal kinase.

adenosine receptors within the various pituitary cell types and also suggested that the physiologically relevant subtype in the anterior pituitary may be the A_{2B} receptor. These findings are illustrated in Fig. 1.

Effects of adenosine on pituitary hormone release

The effects of adenosine on hormone secretion within the pituitary gland have been studied extensively. As long ago as 1976, adenosine was shown to stimulate GH secretion and reverse the apomorphine inhibition of PRL synthesis in rat hemipituitaries (Hill *et al.* 1976). However, these effects were only apparent at very high concentrations of the nucleoside and were mimicked by guanosine. Others later showed that adenosine could stimulate the release of PRL when given peripherally (Stewart & Pugsley 1985) or centrally (Ondo *et al.* 1989), but not of luteinising hormone (LH) (Ondo *et al.* 1989) or thyrotrophin (TSH) (Di Renzo *et al.* 1990). The effects of xanthines, such as caffeine, theobromine and denbufylline, which act as adenosine receptor antagonists, have also been examined in the pituitary gland. These compounds have been shown to increase ACTH and corticosterone, while lowering TSH and GH concentrations (Spindel *et al.* 1983,

Nicholson 1989, Hadley *et al.* 1996, Kumari *et al.* 1997). Xanthines, however, also act as potent inhibitors of phosphodiesterases and it is likely that much of their activity in the pituitary gland relates as much to inhibition of these enzymes as to blockade of adenosine receptors (Hadley *et al.* 1996, Kumari *et al.* 1997). Nevertheless, these studies suggested that adenosine could have significant actions on hormone release in the pituitary gland but the site or mode of action of adenosine and the mediating adenosine receptor subtype(s) were not clear from these experiments.

A number of groups attempted to address these limitations using primary anterior pituitary cell cultures, hemipituitary sections or pituitary cell lines as experimental models. The interpretation of data obtained using these disparate approaches needs to be undertaken with caution as important differences can exist between these models, for example loss of cell–cell interaction in culture, changes in cellular composition or alteration in receptor number or affinity in cell lines as has been reported previously for the D_2 dopamine receptor in GH_3 cells (Johnston *et al.* 1991). Despite these limitations, a number of groups were able to show that adenosine inhibits GH, PRL (Dorflinger & Schonbrunn 1985, Delahunty *et al.* 1988, Scorziello *et al.* 1993), LH and follicle-stimulating hormone (FSH)

Figure 2 Effects of adenosine receptor activation on hormone secretion in the anterior pituitary gland. Adenosine has been shown to stimulate ACTH release although it is unclear whether this is a direct effect or an indirect effect via folliculostellate cells. The identity of the A_2 receptor subtype that mediates ACTH or TSH secretion is not known. In studies involving clonal pituitary tumour cell lines, the A_1 receptor mediates inhibition of GH and PRL secretion but in hemipituitary sections the effects on PRL release are variably stimulatory or inhibitory. The A_1 receptor has been reported to inhibit LH and FSH release in rat hemipituitaries, whereas the effects on TSH in a similar model are either inhibitory (A_1) or stimulatory (A_2). In each case, with the exception of studies on A_1 receptor-mediated inhibition of GH and PRL in cell lines, direct or indirect actions of adenosine on hormone release have not been established. Adenosine has also been shown to be an agonist at the growth hormone secretagogue receptor but failed to modify GH release *in vitro*.

(Picanco-Diniz *et al.* 1989, Yu *et al.* 1998) secretion in the pituitary via the A_1 receptor. However, these effects on PRL were particularly controversial since other investigators showed that low concentrations of adenosine, again acting at the A_1 receptor, stimulated PRL secretion (Picanco-Diniz *et al.* 1989, Yu *et al.* 1998). As well as its stimulatory effects on ACTH release (Scaccianoce *et al.* 1989, Anand-Srivastava *et al.* 1989), adenosine, via the A_{2A} receptor, has also been shown to stimulate PRL and TSH secretion (Kumari *et al.* 1999). These apparent discrepancies in A_1 and A_{2A} receptor action are not easily reconciled as similar techniques were used in each case. Nevertheless, these studies highlight the importance of using homogeneous cell populations or cell lines as well as mixed cell cultures and intact tissue, and reinforce the value of obtaining a detailed description of the expression patterns of adenosine receptors in endocrine and non-endocrine pituitary cells.

More recently, adenosine was identified as an agonist of the growth hormone secretagogue (GHS) receptor (Tullin *et al.* 2000) following HPLC fractionation of crude hypothalamic extracts and screening of the fractions for activity in cells which expressed recombinant GHS receptor. *In vitro* studies, however, failed to show any effects of adenosine or R-PIA on GH secretion either basally or during stimulation by a growth hormone secretagogue (growth hormone releasing peptide (GHRP)-6) or growth hormone releasing hormone (GHRH). These findings raise doubts as to the physiological relevance of adenosine as a stimulator of GH release in the pituitary gland. A summary of the known actions

of adenosine receptors on hormone production in the anterior pituitary is shown in Fig. 2.

Effects of adenosine on pituitary cell proliferation: evidence for opposing actions on endocrine and folliculostellate cells

In contrast to the studies of adenosine on hormone release, there are few reports of its actions on proliferation of pituitary cells. Several G protein-coupled receptors, including somatostatin, thyrotrophin releasing hormone (TRH) and dopamine receptors, have been shown to mediate the inhibition of growth in pituitary tumour-derived cells. Navarro and colleagues (1997) showed that adenosine binding to the A_1 receptor altered cell cycle kinetics but not cell number in GH₄ cells. This change in the cell cycle was attributed to a more rapid progression from G₀/G₁ to S phase and from S phase to G₂/M phase without accelerating initiation of a new cycle. On the basis of these findings, and their observation that cells in G₀/G₁ were more refractory to TRH-induced PRL release than the overall population, the authors proposed that adenosine acts in the pituitary to permit cells to remain longer in a state (G₀/G₁) more refractory to secretory signals. Lewis and colleagues (1997) also examined the effects of adenosine on cell proliferation in GH₃ cells. They showed that high concentrations of adenosine (100 μ M or above) inhibited the proliferation of these cells via an intracellular rather than a receptor-mediated site of action. This inhibition was reversed by the adenosine transport

function of folliculostellate cells is unclear, recent evidence suggests that they form a three-dimensional network in the anterior pituitary and provide a critical link between central and peripheral stimuli and endocrine cell function (Fauquier *et al.* 2001). A number of investigators have highlighted the importance of these cells in regulating immune–neuroendocrine interactions and adenosine has previously been shown to increase IL-6 release from primary anterior pituitary cell cultures (Ritchie *et al.* 1997). Our recent studies have examined this relationship in more detail. We have shown that adenosine, via the A_{2B} receptor, potently stimulates IL-6 release from folliculostellate cell cultures, mediated via adenylate cyclase and phospholipase C coupled to p38 MAP kinase (unpublished observations). Similar but much less potent effects were seen on vascular endothelial growth factor (VEGF) release and may represent an autocrine effect of IL-6 (Renner *et al.* 1997). The production of both IL-6 and VEGF was reversible by co-incubation with dexamethasone. Systemic and locally generated IL-6 is known to be a potent stimulator of ACTH (and GH, PRL and gonadotrophin) release in the pituitary gland (Spangelo *et al.* 1989, Mastorakos *et al.* 1994) and these results therefore suggest that adenosine may have an important role in activation of the pituitary gland in regulating the response of the hypothalamic–pituitary–adrenal axis to inflammation. Whether secretion of other members of the gp130 family of cytokines, which are known to be expressed in folliculostellate cells (Perez Castro *et al.* 2000), is similarly induced by adenosine is unclear at present and represents an important area for future research.

Adenosine exerts potent anti-inflammatory effects in other tissues and disease states. In rheumatoid arthritis, for example, part of the anti-inflammatory actions of methotrexate and sulphasalazine are mediated via enhanced production and/or action of adenosine, and specific immunosuppressive functions attributed to the nucleoside include inhibition of IL-2, tumour necrosis factor (TNF)- α and macrophage inflammatory protein-1 α synthesis (Spychala 2000). We hypothesise that pituitary folliculostellate cells respond to immune stimuli and endotoxins by releasing adenosine (Yu *et al.* 1998), perhaps via toll-like receptor-4, resulting in autocrine stimulation of IL-6 and VEGF, which, in turn, modulate inflammatory responses and increase steroidogenesis.

The immunomodulatory properties of adenosine, together with its cytoprotective and growth-promoting functions in some cells (Spychala 2000), has led to suggestions that it could play a role in solid tumour growth. In this context, the potent effects of adenosine on folliculostellate cell IL-6 and VEGF production raise an intriguing possibility that the nucleoside could have a role in contributing to the maintenance of pituitary tumour cell growth. Hypoxia is a major stimulus to the release of adenosine. Under these circumstances micromolar con-

centrations of the nucleoside typically accumulate. Many solid tumours are characterised by their hypoxic cores and it is conceivable that adenosine and other purines accumulate in the interstitium of such tumours in high concentration. In this regard it is interesting to note that adenosine monophosphate, the immediate precursor of adenosine, is secreted by pituitary tumours (Lewis *et al.* 1996). Should the distribution and relative affinities of A₁ and A_{2B} adenosine receptors be mirrored *in vivo* then A_{2B} adenosine receptors on folliculostellate cells, which can accumulate in a transition zone at the edge of pituitary adenomas (Farnoud *et al.* 1994), may be activated. This could result not only in folliculostellate cell growth but also contribute to an increased proliferation of vascular and endocrine cells in response to the paracrine release of VEGF and IL-6 respectively (Sawada *et al.* 1995). However, although pituitary adenomas may be less vascular than the normal gland (Turner *et al.* 2000) and therefore more prone to the effects of hypoxia, they are generally very small and the mechanisms outlined above may only be of minor importance in their overall growth. These potential mechanisms are illustrated in Fig. 3.

In summary, recent advances using a complementary range of methodologies have provided a more detailed description of the cell-specific distribution of adenosine receptors within the pituitary gland, in turn generating new insights into their function. The coexistence of more than one adenosine receptor subtype in the pituitary may enable the common agonist adenosine to activate multiple signalling pathways. As the A₁ and A_{2B} adenosine receptors are respectively negatively and positively coupled to adenylate cyclase, this may allow reciprocal control of this signalling pathway. In this regard, if the adenosine receptor subtypes in the pituitary have different affinities, then the local concentrations of adenosine under physiological and pathological conditions are likely to be of paramount importance in determining their activation. Dissecting the factors involved in controlling these concentrations, such as extracellular nucleotidases (Spychala 2000) and molecules regulating purine release from pituitary cells (Chen *et al.* 1995), is likely to be of great significance in improving our understanding of the role of adenosine in the pituitary.

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