Allosteric interactions at adenosine A₁ and A₃ receptors: new insights into the role of small molecules and receptor dimerization

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The purine nucleoside adenosine is present in all cells in tightly regulated concentrations. It is released under a variety of physiological and pathophysiological conditions to facilitate protection and regeneration of tissues. Adenosine acts via specific GPCRs to either stimulate cyclic AMP formation, as exemplified by Gₛ-protein-coupled adenosine receptors (A₂A and A₂B), or inhibit AC activity, in the case of G₁/o-coupled adenosine receptors (A₁ and A₃). Recent advances in our understanding of GPCR structure have provided insights into the conformational changes that occur during receptor activation following binding of agonists to orthosteric (i.e. at the same binding site as an endogenous modulator) and allosteric regulators to allosteric sites (i.e. at a site that is topographically distinct from the endogenous modulator). Binding of drugs to allosteric sites may lead to changes in affinity or efficacy, and affords considerable potential for increased selectivity in new drug development. Herein, we provide an overview of the properties of selective allosteric regulators of the adenosine A₁ and A₃ receptors, focusing on the impact of receptor dimerization, mechanistic approaches to single-cell ligand-binding kinetics and the effects of A₁- and A₃-receptor allosteric modulators on in vivo pharmacology.

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Abbreviations

ADA, adenosine deaminase

Introduction

Adenosine is a reactive metabolite that has a major role in regulating a number of physiological and pathological processes, including inflammation, pain, hypoxia and cardiovascular regulation (Fredholm et al., 2011). Adenosine acts via four specific GPCRs, which have been denoted as adenosine A₁, A₂A, A₂B and A₃ receptors (Alexander et al., 2013; Fredholm et al., 2011). The A₁ and A₃ receptors preferentially couple to G₁/o proteins and have an inhibitor action on AC activity, while the A₂A and A₂B receptors couple to Gₛ proteins and stimulate cyclic AMP formation (Jacobson, 2009; Fredholm et al., 2011; Muller and Jacobson, 2011). The crystal structure of the A₂A receptor in both antagonist (Jaakola et al., 2008) and agonist (Xu et al., 2011) bound conformations has recently been solved. Numerous selective agonists and antagonists for each adenosine receptor subtype are now available for the study of receptor function (reviewed in Jacobson, 2009; Fredholm et al., 2011; Muller and Jacobson, 2011). In the case of the G₁/o-coupled adenosine receptors (A₁ and A₃) reviewed here, a number of compounds are undergoing evaluation for disease indications (Muller and Jacobson,
of adenosine A1 receptors in mediating localized analgesia in nucleotides and subsequent production of adenosine follow-

2001; Chiavegatta

phosphodiesterase and ecto-5′

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2004) and it is known that extracellular cyclic AMP can be

intracellular cyclic AMP can be released from cells in response

sequence of several pathways (Fredholm et al., 2011). It can be

formed intracellularly following various metabolic processes

and be exported from cells via membrane transporters, or it

can be formed in the extracellular space from adenine nucleo-
tides released from cells. Once ATP or ADP is released, the

nucleotide is broken down by nucleoside triphosphate

diphosphohydrolases (e.g. CD39) and then ecto-5′-
nucleotidase (CD73) to adenosine (Fredholm et al., 2011; Knapp

et al., 2012). It is well known that neurons and plate-

lets can store and release ATP and ADP, respectively, in

response nerve stimulation and platelet activation. However,

more recently, there has been a growing awareness that the

intracellular second messenger cyclic AMP may also be a

source of extracellular adenosine in many cell types. Thus,

intracellular cyclic AMP can be released from cells in response

to receptor stimulation (McCrea and Hill, 1993; Baker et al.,

2004) and it is known that extracellular cyclic AMP can be

rapidly converted to adenosine via the action of ecto-

phosphodiesterase and ecto-5′-nucleotidase (Dubey et al.,

2001; Chiavegatta et al., 2008; Goedeke, 2008).

The intricacies of localized extracellular release of adenine

nucleotides and subsequent production of adenosine follow-


regulated at the level of its target receptor is via drugs that

bind to an allosteric site on the receptor and act as allosteric

modulators to enhance or inhibit the binding and/or func-
tion of adenosine. Here, we review the properties of various

small-molecule allosteric regulators of the adenosine A1 and

A3 receptors focusing on the impact of receptor dimerization,

mechanistic approaches to single-cell ligand-binding kinetics

and the effects of A1- and A3-receptor allosteric modulators on

in vivo pharmacology.

Allosteric regulation of GPCRs

GPCRs comprise the largest family of transmembrane pro-
teins and represent major targets for drug discovery (Williams and

and Hill, 2009; Roth and Marshall, 2012). Considerable

advances in our knowledge of GPCR structure have been

made recently (Jaakola et al., 2008; Chien et al., 2010; Chung

et al., 2011; Rasmussen et al., 2011) and this has led to sig-

nificant insights into the conformational changes that occur

during receptor activation in response to agonists that act at

the same site (orthosteric) as the endogenous hormone or

neurotransmitter (Chung et al., 2011; de Graaf et al., 2011;

Rasmussen et al., 2011). However, over the past decade, there

has been an increasing acceptance that drugs can also bind to

to a topographically distinct site (allosteric) on the GPCR

protein and elicit a conformational change that can lead to a

change in the affinity or efficacy of a ligand occupying the clásical

orthosteric binding site (Figure 1A; May et al., 2007;

Kenakin, 2009, 2012; Keov et al., 2010). This suggests that

GPCRs are able to bind more than one ligand simultaneously

(i.e. both an allosteric and an orthosteric ligand; May et al.,

2007; Kenakin, 2009, 2012; Keov et al., 2010). Various math-

eatical models have been developed to explain these phe-
nomena, but key features of an allosteric mechanism of

action are that the effect is saturable, can depend on the

specific ligand occupying the orthosteric site (probe depend-

ence) and provides scope for both positive and negative

effects on ligand binding and/or function (May et al., 2007;

Kenakin, 2009, 2012; Keov et al., 2010).

Some of the earliest allosteric modulators were discovered

for the adenosine A1 receptor (Bruns and Fergus, 1990; Bruns

et al., 1990; Göblyöös and Ijzerman, 2011; Kimatrai-Salvador

et al., 2012). PD 81,723 has become a reference allosteric

enhancer for the A1 receptor. Early studies demonstrated that

PD 81,723, which has A1-receptor antagonist properties at

high concentrations, was able to increase the binding of an

orthosteric agonist radioligand at lower concentrations of PD

81,723 to enhance the functional activation of the A1 recep-
tor in the brain (Janusz and Berman, 1993) and cardiovascular

tissues (Amoah-Apraku et al., 1993) and to slow down the

dissociation of the agonist radioligand from the A1 receptor

(Bruns and Fergus, 1990; Bruns et al., 1990); the latter effect

being indicative of an allosteric mechanism of action (see

below; May et al., 2007; Keov et al., 2010; Göblyöös and

Ijzerman, 2011). Furthermore, recent studies using site-
directed mutagenesis have indicated that the allosteric

binding site for PD 81,723 may reside within extracellular

loop 2 of the adenosine A1 receptor (Peeters et al., 2012).

Selective allosteric enhancers of agonist binding have also

been described for the adenosine A1 receptor (Gao et al., 2001;

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The impact of an allosteric modulator is not, however, restricted to the binding and function of orthosteric agonists. For example, the food dye Brilliant Black BN is able to act allosterically to reduce the affinity of particular adenosine A1- and A3-receptor antagonists (e.g. xanthine amine congener) for the orthosteric site without altering the ability of agonists to interact with these two receptors (May et al., 2010a). This is a good example of probe dependence where the effect observed differs, depending on the nature of the ligand occupying the orthosteric site. In addition to small molecules exerting allosteric influences on GPCRs, there is considerable evidence that sodium ions can also mediate allosteric effects on a range of GPCRs, including both the adenosine A1 and A3 receptors (Liu et al., 2012). In the case of A1 and A3 receptors, a highly conserved aspartate residue in transmembrane region 2 of each receptor (Asp2,50) has been implicated in the allosteric actions of sodium ions. Mutation of this residue to alanine or asparagine largely abolishes the effect (Barbhaiya et al., 1996; Gao et al., 2003). In a recent high-resolution crystal structure of the adenosine A2A receptor, the precise location of the sodium ion and its associated water cluster has been identified and shown to interact with Asp2,50 (Liu et al., 2012).

Recent studies with imidazoquinolinamine allosteric enhancers (e.g. LUF5999, LUF6000 and LUF6001) of the adenosine A3 receptor have shown that they have differing effects on the affinity and efficacy of a selective A3-agonist Cl-IB-MECA (Gao et al., 2011). This illustrates the independence of allosteric actions on binding affinity and efficacy. Furthermore, the allosteric modulation of orthosteric agonist efficacy was dependent on the intracellular signalling response being measured. This suggests that the allosteric modulation of agonist efficacy may be functionally biased (Gao et al., 2011). Functional selectivity of orthosteric and allosteric ligands has also been investigated for the adenosine A1 receptor (Cordeaux et al., 2004; Valant et al., 2010; Langemeijer et al., 2013). The magnitude of positive allosteric modulation of the 2-amino-3-benzoylthiophene adenosine A1-receptor allosteric enhancer, VCP520, varied between pathways (Valant et al., 2010). This is an example of an
allosteric modulation engendering functional selectivity in the actions of orthosteric ligands (Valant et al., 2010). These studies highlight the ability of allosteric ligands to further ‘fine-tune’ orthosteric ligand responses. Signalling bias from GPCRs is a concept that has developed considerably over the last few years as knowledge that GPCRs can regulate signalling pathways independently of heterotrimeric G protein has become available (e.g. β-arrestin pathways; Kenakin, 2012; Whalen et al., 2011). Thus, it is clear that activation of β-arrestin pathways are not only associated with desensitization and receptor internalization but can also change the signalling pathways that are activated. Furthermore, specific agonists appear to be able to direct signalling to different pathways via the same cell surface receptor. Some of the best evidence for this has come from the β₂-adrenoceptor field, where certain β-blockers (e.g. propranolol) can have an inverse agonist effect of Gₛ-mediated signalling pathways, but an agonist action on MAP kinase (Azzi et al., 2003; Baker et al., 2003). The concept of biased signalling, however, is a natural extension of allosterism (Figure 1B). The intracellular signalling proteins (e.g. heterotrimeric G proteins of β-arrestin) bind to the GPCR at a site distinct from the orthosteric binding site. As a consequence, they can be considered as allosteric regulators (in this case, proteins) that can have a reciprocal effect on ligand binding (or coupling in the case of the protein) and lead to altered affinity and efficacy for particular agonists (Kenakin, 2012). In many ways, therefore, biased signalling is a natural consequence of a key feature of allosterism, namely probe dependence (Figure 1B).

The ability of receptor-associated proteins to act as allosteric modulators of ligand binding and efficacy can be extended to neighbouring receptors that form homo- or heterodimers or higher order oligomers (Figure 1C). For example, we have recently provided evidence for negative cooperativity across the dimer interface of an adenosine A₁-receptor homodimer (May et al., 2011). In this case, binding of an orthosteric ligand to one protomer (monomeric component) of the homomorphic complex can markedly alter the affinity of a ligand binding to the second protomer (May et al., 2011). Evidence is also accumulating that the adenosine A₁ receptor can form heterodimers with P₁Y receptors, adenosine A₂A receptors, β₁- and β₂-adrenoceptors to influence orthosteric ligand binding and/or intracellular signalling (Suzuki et al., 2006; Chandrasekera et al., 2013; Cristovao-Ferreira et al., 2013; Franco et al., 2013). In addition to partner receptors within oligomeric complexes, other extracellular proteins can also bind to GPCRs and mediate allosteric influences. For example, adenosine deaminase (ADA), which is a key enzyme catalysing the deamination of adenosine, can be released from cells and bind to cell surface proteins and act as an ectoenzyme (Gracia et al., 2013). One of the proteins that bind ADA is the adenosine A₁ receptor (Ciruela et al., 1996; Gracia et al., 2013). The association of ADA with the adenosine A₁ receptor can lead to enhanced agonist affinity and efficacy (Gracia et al., 2008; 2013). The consequence of a metabolic enzyme for the endogenous activator being associated with the cell surface adenosine receptor, which can enhance affinity and efficacy, is therefore likely to amplify local signalling while limiting the duration of action (and spread of activity) due to its local metabolic activity.

Overview of small-molecule allosteric regulators acting on the adenosine A₁ and A₃ receptors

The therapeutic potential of allosteric regulators that can amplify or modulate the local actions of adenosine is clear and efforts are in progress to develop these reagents for a wide range of GPCRs. The development of allosteric modulators targeted at the adenosine receptor family has recently been comprehensively reviewed (Göblöös and Ijzerman, 2011; Jacobson et al., 2011). For brevity, we will focus our discussion on the medicinal chemistry of allosteric modulators specifically mentioned within this review (Figure 2). As stated earlier, PD 81,723 was one of the key compounds originally described in back-to-back papers from the Parke-Davis Pharmaceutical Research Division (Bruns and Fergus, 1990; Bruns et al., 1990) recounting the identification of the first allosteric regulators of adenosine A₁-receptor binding. The chemical series was originally identified from the Parke-Davis compound bank via a 300-ligand adenosine A₁ binding screen. While the 2-amino-3-benzoylthiophene chemical scaffolds had been originally synthesized as intermediates for benzodiazepine-like compounds (Tinney et al., 1974), recognition of their adenosine antagonist activity prompted a more thorough analysis of this privileged chemical template (Bruns and Fergus, 1990). From this medicinal chemistry study, second-generation compounds were unexpectedly found to increase the specific binding of [³H]N-α-cyclohexyladenosine to rat brain membranes. This, in turn, resulted in the synthesis of further compounds to identify pertinent structure–activity relationships and, in so doing, identified PD 81,723 as a key analogue displaying a significantly improved allosteric profile (Bruns et al., 1990). This core structure has been further modified by a number of groups thereby developing a robust structure activity relationship profile and a series of ligands with comparable or more favourable allosteric activity (van der Klein et al., 1999; Kourounakis et al., 2000; Baraldi et al., 2003, 2004; Nikolakopoulos et al., 2006; Romagnoli et al., 2008; Valant et al., 2010). One particularly successful manipulation centred on removal of the 4- and 5-methyl groups and installation of substituted phenyl rings back into these positions of PD 81,723 (Aurelio et al., 2008). It was interesting to note that the most efficacious compound possessed no substituent in the 5-position and this lead to further exploitation of this observation through the synthesis and evaluation of the next generation of ligands, which identified VCP520 as a potent allosteric enhancer of A₁-receptor-mediated signalling (Aurelio et al., 2009).

In a similar fashion as the discovery of PD 81,723, the lead compounds recognized as allosteric modulators of the adenosine A₃ receptor were identified from screening diverse chemical libraries in binding assays at this receptor subtype (Jacobson et al., 2011). In this instance, certain lead molecules were shown to increase the level of binding of [¹²⁵I]AB-MECA (Gao et al., 2001; 2002). Key molecular scaffolds that supported allosteric modulation at the adenosine A₃ receptor were identified as 3-(2-pyridinyl)isoquinolines (e.g. VUF5455) and 3H-imidazo-[4,5-c]quinolin-4-amines (e.g. LUF5999, LUF6000 and LUF6001). With regard to the former, further exploration of the 3-(2-pyridinyl)isoquinoline scaffold...
revealed a complex situation where some members were pure antagonists of the orthosteric binding site, for example, VUF5455 itself (Heitman et al., 2009), consequently rendering them not particularly useful as future therapeutics. However, the imidazoquinolinamines fared better in this respect; the original molecule DU124183 (Gao et al., 2002) was further modified at the 2- and 4-positions and numerous resultant derivatives displayed potentiation of the maximum efficacy of Cl-IB-MECA at the A3 receptor (Göblyös et al., 2006). Indeed, LUF6000 was shown to enhance agonist efficacy in a functional assay and decrease agonist dissociation rate without influencing agonist potency. This was postulated to be a result of its experimentally observed decreased interaction with the orthosteric binding site on the adenosine A3 receptor. As previously mentioned, a more thorough analysis of this and related ligands (LUF5999 and LUF6001) identified that these imidazoquinolinamine allosteric enhancers displayed differing effects on the affinity and efficacy of Cl-IB-MECA at the A3 receptor (Gao et al., 2011). In a related study, with the intention of overcoming the issues associated with the orthosteric antagonism shown by the 3-(2-pyridinyl)isoquinolines, a series of ring opened imidazoquinolinamines were synthesized to afford a range of 2,4-disubstituted quinolines as a new class of allosteric enhancers at the A3 receptor (Heitman et al., 2009). Rewardingly, the best compound (LUF6096) was not only able to allosterically enhance the binding of CI-IB-MECA to a similar level as LUF6000 but it also displayed negligible orthosteric affinity for any of the adenosine receptor subtypes. These compounds have begun to be used in mechanistic studies to identify the basis of these allosteric effects on efficacy and affinity and the extent to which these two effects are related.

**Mechanistic insights from single-cell ligand-binding kinetics**

Allosteric interactions are a mode of communication between distal binding sites. Intra- and intermolecular GPCR allosterism with transmembrane proteins and allosteric small molecules can generate a unique spectrum of resting and/or active distribution of GPCR conformations, which, in turn, can significantly influence the pharmacology of orthosteric and/or allosteric ligands. Typically, GPCR allosterism changes the properties of conformationally linked binding sites and therefore the association and/or dissociation kinetics of the cognate orthosteric ligands (May et al., 2007; Smith and Milligan, 2010). Orthosteric ligand affinity is described by the ratio of the association to dissociation rates, and as such, an allosteric interaction that alters orthosteric ligand affinity does so by mediating a change in one or both of these parameters. Dissociation kinetic assays can be used as a powerful mechanism to validate an allosteric mechanism of action of a ligand since orthosteric and allosteric ligands must interact with the receptor simultaneously to change the dissociation kinetics of a labelled orthosteric ligand. Plotting the dissociation rate of labelled orthosteric ligand in the presence of
a range of interacting ligand concentrations provides a concentration–response relationship of a purely allosteric effect (Kostenis and Mohr, 1996). Furthermore, the midpoint of this curve provides an estimate of affinity of the orthosteric ligand for the allosteric modulator occupied receptor.

Typically, dissociation kinetic studies investigating intramolecular allosterism use isotopic dilution. That is, the influence of an allosteric ligand on the dissociation kinetics of an orthosteric radiolabelled probe is assessed in the presence of a saturating concentration of a second competitive orthosteric ligand (Bruns and Fergus, 1990a; Ellis et al., 1991; Lee and el-Fakahany, 1991; Lazareno and Birdsall, 1995; Christophopoulos et al., 1997; Gao et al., 2001; Avlani et al., 2004; Dowling and Charlton, 2006). A key assumption required for interpreting such dissociation kinetic studies, however, is that the second ligand does not alter the rate of radioligand dissociation. This assumption is consistent within a theoretical framework describing competitive interactions between compounds at a monomeric receptor; however, more complex interactions resulting from multistep ligand binding (Swaminath et al., 2004; Illen et al., 2009) or receptor dimerization (Christopoulos and Kenakin, 2002; Springael et al., 2006; Han et al., 2009; May et al., 2011) could lead to a change in the radioligand dissociation rate. Recently, the binding kinetics of a fluorescent adenosine derivative was determined in the absence and presence of allosteric modulators at the adenosine A1 and A3 receptor in live single cells (May et al., 2010b). Importantly, these studies were performed using a closed perfusion system that enabled rapid removal of free ligand (May et al., 2010a) and therefore assessed the dissociation kinetics under ‘infinite dilution’ conditions in the absence of a saturating concentration of competitive orthosteric ligand. Similar to the previous studies, which used isotopic dilution to promote orthosteric radioligand dissociation, PD 81,723 significantly retarded the dissociation of the fluorescent adenosine derivative from the adenosine A1 receptor (May et al., 2010b). In contrast, VUF5455, which has previously been demonstrated to decrease the rate of agonist dissociation from the adenosine A1 receptor (Gao et al., 2001), was found to significantly enhance the fluorescent agonist dissociation rate (May et al., 2010b). This discrepancy may reflect the different orthosteric agonist probes used in the different studies and therefore the ability of allosteric modulators to be highly probe-dependent (May et al., 2007). Alternatively, the difference could reflect a more complex receptor arrangement than a non-interacting monomer, that is, a dimer or higher order oligomer (see below).

Traditionally, GPCRs have been considered to exist and function as monomeric proteins. However, it is now known that GPCRs can form homodimers, heterodimers and/or higher order oligomers (Smith and Milligan, 2010). Non-visual GPCRs can be classified into three families, A–C. Family C GPCRs are known to function as obligate dimers (Kuszak et al., 2009). However, evidence suggests that cell surface complexes of family A GPCRs may display a distinct profile of functional properties relative to their monomeric counterparts. For example, dimerization and/or oligomerization may influence signal transduction efficiency, receptor desensitization and/or the ligand preference for coupling to particular downstream signalling cascades (May et al., 2007; Smith and Milligan, 2010; Franco et al., 2013). Furthermore, a recent study provided evidence for communication between simultaneously bound orthosteric sites on homodimeric dopamine D2 receptors (Urizar et al., 2011). As such, a ligand bound to one protomer can modulate ligand function and/or affinity at a second interacting protomer. This may lead to complex pharmacology and/or the potential for dimeric species to elicit specific signalling events with unique pharmacological properties.

The fundamental premise of intra- and intermolecular allosteric modulation is based on conformational rearrangements; therefore, a wealth of information can be gained through assessing ligand-binding kinetics under different conditions. Dissociation kinetic studies provided the first evidence for homodimerization of a family A GPCR, the β-adrenergic receptor. This study used an ‘infinite dilution’ approach to detect a change in the dissociation kinetics of the radiolabelled orthosteric ligand, [3H](-)alprenolol, in the absence and presence of unlabelled (-)alprenolol. The increased dissociation rate in the presence of unlabelled orthosteric ligand, (-)alprenolol, was suggestive of negatively cooperative interactions across a β-adrenergic homodimeric interface (Limbird et al., 1975). Intermolecular cooperativity between orthosteric binding sites has since been established for a number of additional GPCRs, including adenosine and muscarinic ACh receptor subtypes (Briddon et al., 2008; Casadó et al., 2010; Hern et al., 2010; Pisterzi et al., 2010; Hu et al., 2012; May et al., 2011). At the adenosine A1 and A3 receptors, dissociation kinetic analysis has been employed as a powerful method to detect intermolecular allosterism, that is, cooperative interactions across a homodimeric interface (May et al., 2011). In contrast to the adenosine A1 receptor, highly cooperative interactions were observed under ‘infinite dilution’ conditions between the fluorescent adenosine derivative and orthosteric agonists and antagonists at the adenosine A3 receptor. Figure 3 shows an example of the effect of increasing concentrations of the endogenous orthosteric ligand adenosine on the dissociation kinetics of a fluorescent adenosine analogue from the human adenosine A3 receptor. In marked contrast, adenosine had a much less marked effect on the dissociation kinetics of the fluorescent ligand from the human adenosine A3 receptor (May et al., 2011). Importantly, the intermolecular allosterism was significantly decreased upon co-expression of a non-binding adenosine A3–receptor mutant, supporting the suggestion of cooperative interactions across the dimeric interface of cell surface adenosine A3 receptors (May et al., 2011). These studies add strength to the suggestion that the discrepancy observed between the influence of the allosteric modulator, VUF5455, in dissociation kinetic studies using isotopic dilution as compared to infinite dilution may reflect the ability of adenosine A3 receptors to form interacting homodimers and/or higher order oligomers. In keeping with this hypoth-
esis, if a non-fluorescent orthosteric ligand (e.g. MRS1220) is added simultaneously with a derivative of VUF5455 (VUF5645), then this allosteric compound then produces a slowing down of the dissociation kinetics of the fluorescent adenosine analogue (Figure 4).

**Impact of A1- and A3-receptor allosteric modulators on in vivo pharmacology**

Adenosine A1- and A3-receptor ligands (both agonists and antagonists) have been developed for a number of potential therapeutic indications (Muller and Jacobson, 2011). These are summarized in Table 1. The best developed indications appear to be for agonists where A1-receptor agonism may have utility in angina, neuropathic pain, paroxysmal supraventricular tachycardia and ischaemia (Griffin et al., 2003; Morrison et al., 2006; Albrecht-Kupper et al., 2012; Tendera et al., 2012), and A3-receptor agonists may have benefit in liver cancer, rheumatoid arthritis, autoimmune inflammatory disease, dry eye and cardiac ischaemia (Table 1; Bar-Yehuda et al., 2011; Cohen et al., 2011; Fishman et al., 2012). With regard to cancer, it is interesting that A3 receptors appear to be overexpressed in certain cancers (e.g. breast and colon cancer) compared to normal cells (Gessi et al., 2004; Madi et al., 2004; Bar-Yehuda et al., 2008; Fishman et al., 2012).

Studies with genetically altered mice have also suggested a role of A1 receptors in pain (Sowa et al., 2010) and ischaemia (Matherne et al., 1997) and for A3 receptors in cardiac ischaemia (Ge et al., 2006) and mast cell degranulation (Salvatore et al., 2000). In the latter case, it is worth pointing out that functional A3 receptors appear to be absent from human mast cells (Fredholm et al., 2011). However, although selective A3-receptor activation is cardio-protective in wild-type mice and those overexpressing the A3 receptor, adenosine A3-receptor gene deletion generates an ischaemia-tolerant phenotype that might be indicative of compensatory changes (Harrison et al., 2002).

As mentioned in the introductory remarks, the ubiquitous distribution of adenosine receptors and the potential for serious side effects via the target receptor in a different organ or cell type can limit their utility. For example, in many non-cardiac therapeutic applications of A1-receptor agonists, the potential for major side effects due to A1-receptor actions in the heart will be seriously limiting. This may be particularly true in the case of adenosine A1-receptor agonists that may have potential utility in the treatment of CNS diseases, such as epilepsy (Mares, 2010; Klaft et al., 2012). This has led to the development of partial agonists (e.g. capadenoson; Albrecht-Kupper et al., 2012; Tendera et al., 2012) that may

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**Figure 3**

Adenosine mediates a significant enhancement in the dissociation of 30 nM ABA-X-BY630 from the human adenosine A3 receptor. (A) Dissociation of a fluorescent adenosine analogue, ABA-X-BY630 (30 nM), from CHO-A3 cells in the absence or presence of adenosine (1 μM; 10 μM; 100 μM). (B) Concentration dependence of the changes in koff of 30 nM ABA-X-BY630 from CHO-A3 cells in the absence and presence of adenosine. Data points are expressed as mean ± SEM from 3–11 separate experiments; each replicate represents the average fluorescence at the plasma membrane of 10 individual cells. Data taken from May et al. (2011).

**Figure 4**

The influence of the competitive antagonist, MRS1220, and/or the allosteric ligand, VUF5645, on the dissociation kinetics of the fluorescent adenosine derivative, ABA-X-BY630. ABA-X-BY630 (30 nM) dissociation in the absence and presence of a 1 μM MRS1220, 1 μM VUF5645, or 1 μM MRS1220 and 1 μM VUF5645. Representative data performed in duplicate; each replicate represent the average fluorescence at the plasma membrane of 10 individual cells.
Interestingly, the A1-receptor allosteric enhancer PD 81,723 has been reported in the CNS where administration of PD 81,723 has been achieved with PD 81,723 as a consequence of amplifying the restricted increase in adenosine in the kidney following local ischaemia (Park et al., 2012). A similar outcome has been achieved in the absence of significant effects on heart rate (Park et al., 2012). This was achieved in the absence of significant effects on heart rate and BP, which suggests that renal A1-receptor selectivity had been achieved with PD 81,723 as a consequence of amplifying the restricted increase in adenosine in the kidney following local ischaemia (Park et al., 2012). A similar outcome has been reported in the CNS where administration of PD 81,723 can lead to a reduction in hippocampal injury following hyperglycaemic ischaemia in the rat (Meno et al., 2003).

A positive allosteric modulator of the adenosine A1 receptor (LUF6096) has also been shown to have benefit in an in vivo model of myocardial ischaemia/reperfusion injury in the dog (Du et al., 2012). Thus, LUF6096 had no effect on baseline haemodynamic parameters, but pre-treatment with LUF6096 prior to coronary occlusion and during reperfusion produced a marked reduction in infarct size (ca. 50% reduction; Du et al., 2012). An equivalent reduction in the infarct size could also be demonstrated if LUF6096 was administered immediately before reperfusion (Du et al., 2012). These studies collectively indicate that allosteric enhancers of the adenosine A1 and A3 receptors may have great utility as therapeutic strategies to provide selective augmentation of the actions of adenosine released locally in conditions of disease and stress.

Concluding remarks

It is clear that allosteric mechanisms of action provide unique ways to regulate receptor function at a local level to ‘fine-tune’ intracellular signalling. This can be achieved by small molecules (allosteric regulators) or by protein–protein interactions involving signalling proteins (leading to biased signalling) or oligomeric partners (e.g. as a consequence of dimerization). In all cases, these mechanisms provide the potential to exploit the unique pharmacology provided by allosterism to achieve both better cell and tissue selectivity of drug treatments and also interventions with more physiologically relevant kinetic profiles. Novel fluorescent techniques have been able to unravel some of the intricacies involved at the single cell level. However, the therapeutic potential of these actions awaits the clear demonstration of these mechanisms in an in vivo setting.

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Allosterism at adenosine $A_1$ and $A_3$ receptors


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