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Lighting up G protein-coupled purinergic receptors with engineered fluorescent ligands

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ABSTRACT

The use of G protein-coupled receptors fluorescent ligands is undergoing continuous expansion. In line with this, fluorescent agonists and antagonists of high affinity for G protein-coupled adenosine and P2Y receptors have been shown to be useful pharmacological probe compounds. Fluorescent ligands for A_1R, $A_{2A}R$, and A_3R (adenosine receptors) and $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, and $P2Y_{14}R$ (nucleotide receptors) have been reported. Such ligands have been successfully applied to drug discovery and to GPCR characterization by flow cytometry, fluorescence correlation spectroscopy, fluorescence microscopy, fluorescence polarization, fluorescence resonance energy transfer and scanning confocal microscopy. Here we summarize recently reported and readily available representative fluorescent ligands of purinergic receptors. In addition, we pay special attention on the use of this family of fluorescent ligands revealing two main aspects of purinergic receptor biology, namely ligand binding and receptor oligomerization.

This article is part of a Special Issue entitled 'Fluorescent Neuro-Ligands'.

1. Introduction

The purinergic neurotransmission system involves two main extracellular effectors, namely adenosine and adenosine 5′-triphosphate (ATP) (Burnstock, 1972). In the central nervous system (CNS), while adenosine acts as a neuromodulator, ATP also operates as a neurotransmitter. Despite the initial resistance to acceptance of ATP as a genuine extracellular signaling molecule, the existence of consistent physiological effects and specific extracellular enzymes regulating this molecule eventually supported its role as neurotransmitter, and consequently the existence of a purinergic neurotransmission system (Burnstock, 1972). Afterward, ATP was identified as a co-transmitter in peripheral nerves and subsequently as a co-transmitter with glutamate, noradrenaline, GABA, acetylcholine and dopamine in the CNS (Burnstock, 2004). Interestingly, extracellular ATP is quickly hydrolyzed into adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP) and adenosine plus inorganic phosphate and pyrophosphate, through the action of extracellular nucleotidases (ecto-NTs) (e.g. ecto-NTPDases and ecto-5′-nucleotidase) (Zimmermann, 1996). Adenosine, which consists of a purine base (adenine) attached to the 1′ carbon atom of ribose, has been historically considered a retaliatory metabolite modulating a large array of physiological processes (Newby, 1984). Accordingly, it has been postulated that this purine nucleoside is a mediator of metabolic distress, thus having considerable impact on homeostatic cellular functioning. In addition, there is supporting information confirming the involvement of adenosine and ATP in the pathophysiology of the CNS, including neurodegenerative diseases, neuropsychiatric disorders and cancer (i.e. neurogliomas), which have lead to an explosion of interest related to the purinergic neurotransmission system.

Extracellular ATP and adenosine exert their effects via specific plasma membrane receptors. In 1976 purinergic receptors were first defined (Burnstock, 1976), and two years later 2 types of purinoceptors, named P1 and P2 for adenosine and ATP/ADP, respectively, were proposed (Burnstock, 1978). The dual effect of adenosine on cAMP accumulation served as initial subclassification of adenosine receptors into R_i and R_a (Londos et al., 1980), or

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alternatively, A_1 and A_2 adenosine receptors (Van Calker et al., 1979). Nowadays, it is well established that adenosine mediates its actions by activating specific P1 purinergic G protein-coupled receptors (GPCRs), for which four subtypes (i.e. A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) have been identified (Table 1). These receptors have a distinguishable pharmacological profile, tissue distribution and effector coupling (Jacobson and Gao, 2006) (Table 1), and its functioning have been largely studied in the CNS (Sebastião and Ribeiro, 2009). In 1985, P2 receptors were subdivided into P2X and P2Y subtypes on the basis of pharmacology (Burnstock and Kennedy, 1985). While P2X are ligand-gated ion channel receptors, the P2Y subtypes are GPCRs (Burnstock and Williams, 2000). Currently, eight subtypes of the P2Y family (i.e. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) have been cloned and functionally characterized (Abbracchio et al., 2006). Receptors of the P2Y family are variously activated by an expanded list of nucleotides, including ATP, ADP, UTP, UDP and UDP-sugars.

The wide-spread expression of purinergic receptors, in addition to the diverse and abundant roles of these receptors in extracellular signaling, has lead, as commented above, to a great deal of interest for pharmacologists in general and for neuropharmacologists in particular. Indeed, the physiological and pathophysiological consequences of targeting these receptors with specific ligands may offer new opportunities for a purinergic-based pharmacotherapy in various neurological and neuropsychiatric diseases. Accordingly, this scientific interest has prompted a dramatic growth in the purinergic pharmacology field, in which a large number of ligands have been designed and synthesized. Noteworthy, an important contribution to the purinergic field has been the development of selective ligands labeled with fluorescent moieties that remain active at their receptors. Interestingly, these compounds have become powerful tools to address fundamental unmet questions regarding the purinergic receptor biology. For instance, these novel fluorescent ligands of purinergic receptors may permit the visualization of ligand-receptor binding at the cell surface of living cells, or help revealing the existence of purinergic receptor-containing oligomers in native tissue. Accordingly, in the present review, we not only provide an extensive overview of the currently reported fluorescent ligands of purinergic receptors, but also focus on aspects of the biology of adenosine and P2Y receptors to characterize ligand-receptor binding and receptor oligomerization by means of fluorescent ligands.

2. GPCR fluorescent ligands

Fluorescent ligands were introduced in the GPCR field about forty years ago as pharmacological tools to visualize receptors in native conditions and thus boosting the spatial resolution provided by other methodologies (Melamed et al., 1976). The synthesis and purification of these compounds was not easy in those days and thus only accessible to a few researchers. Later on, in the 1990s, the chemistry of fluorescent ligands substantially advanced, and new high-affinity compounds with improved kinetics (i.e. slow dissociation rates) were synthesized (McGrath et al., 1996). Therefore, by using these ligands it was possible the detection of receptors not only at the cell surface level but also within intracellular compartments, thus allowing the study of receptor endocytosis (Lutz et al., 1990). Currently, the use of GPCR fluorescent ligands is undergoing continuous expansion (Ciruela et al., 2014a; Hill et al., 2014; Vernall et al., 2014). In this manner, GPCR-based fluorescent ligands have been successfully introduced in a vast number of techniques, such as flow cytometry (FCM), fluorescence correlation spectroscopy (FCS), fluorescence microscopy (FM), fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), or scanning confocal microscopy (SCM) (Kozma et al., 2013), for the study of receptor physiology and pathophysiology at both the cellular and the subcellular level (Kuder and Kieć-Kononowicz, 2008). In addition, they have been implemented as screening tools in drug discovery (Middleton and Kellam, 2005).

The use of GPCR-selective fluorescent ligands in receptor binding studies has proved to be complementary, and eventually superior, to the classical radioligand-based techniques. Certainly, these compounds show several advantages over conventional radioisotopically-labeled ligands, for instance avoiding the cost of scintillation detection; enhanced safety and reduced costs as no radioisotope use and disposal is needed; 'real-time' readout of the ligand-receptor interaction, which allow kinetic experiments; direct visualization of the receptor localization allowing both local binding and internalization experiments; and finally the possibility of miniaturization, which facilitates a more economical

Table 1
Adenosine receptors and representative fluorescent ligands used to label these receptors.

Receptor	G protein	Transduction mechanisms[b]	Physiological actions	Ligand no.
A_1	$G_{i/o}$[a] $G_{q/11}$ G_s	•**Inhibits**: AC[a] •**Activates**: PLC, AC	Vasoconstriction (Murray and Churchill, 1984); hypothermia and sedation (Anderson et al., 1994); analgesia (Yamamoto et al., 2003); neurotransmitter release (De Lorenzo et al., 2004; Scholz and Miller, 1992); chemotaxis (Schnurr et al., 2004); neuroprotection (MacGregor et al., 1993).	**1** **2** **4** **5** **7**
A_{2A}	G_s[a] G_{olf} $G_{15,16}$ [§]	•**Activates**: AC[a], PLC •**Inhibits**: Ca^{2+} channels	Platelet aggregation inhibition (Varani et al., 2000); vasodilation (Carroll et al., 2006); neurotransmitter release (Popoli et al., 1995); regulation of sensorimotor integration in basal ganglia (Nagel et al., 2003); sleep promotion (Scammell et al., 2001);	**2** **7** **12** **13** **14** **15**
A_{2B}	G_s[a] $G_{q/11}$	•**Activates**: AC[a], PLC	Vasodilation (Kemp and Cocks, 1999); vasoconstriction (Donoso et al., 2005); cytokine production (Zhong et al., 2004); inhibition of cell proliferation (Dubey et al., 2005);	**2** **7**
A_3	$G_{i/o}$[a]	•**Inhibits**: AC[a] •**Activates**: PLC	Mast cell activation (Zhong et al., 2003); preconditioning (Das et al., 2005); coronary vasodilation (Hinschen et al., 2003); regulation of intraocular pressure (Avila et al., 2002); hypotension (Stella et al., 1998).	**2** **6** **7** **9** **10** **11** **16**

[a] Main mechanism of coupling.
[b] AC, adenylyl cyclase; PLC, phospholipase C; PLA2, phospholipase A2; PLD, phospholipase D; GIRKs, G protein-dependent inwardly rectifying K^+ channels.

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