British Journal of Pharmacology (2003) 140, 441–443
www.nature.com/bjp

COMMENTARY

Novel data point to a broader mechanism of action of oxidized ATP: the $P2X_7$ receptor is not the only target

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Oxidized ATP (oATP) is a Schiff-base-forming reagent that has been used for some years as an antagonist at the $P2X_7$ receptor ($P2X_7R$). Preincubation of mononuclear phagocytes with this inhibitor leads to attenuation of several proinflammatory responses triggered by extracellular ATP as well as a few non-nucleotide agonists. Novel data show that oATP reduces NFκB activation and IL-8 release in cells lacking $P2X_7R$, thus suggesting that some anti-inflammatory effects of oATP may not be due to blockade of the $P2X_7R$. This effect of oATP resembles the action of other natural or synthetic Schiff-base-forming reagents with immunomodulatory activity.
British Journal of Pharmacology (2003) **140,** 441–443. doi:10.1038/sj.bjp.0705469

Keywords: $P2X_7$ receptor; extracellular ATP; inflammation; Schiff-base; danger signals

Abbreviations: BzATP, benzoylbenzoyl ATP; oATP, oxidized ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′, 4′-disulfonic acid; P2R, P2 receptor; $P2X_7R$, $P2X_7$ receptor

Receptors for extracellular nucleotides (P2R) are attracting increasing attention in drug development for treatment of pain, inflammation and cancer (Burnstock, 1996; Di Virgilio *et al*., 2001). An intriguing, and appealing, member of the P2R family is the $P2X_7$ receptor ($P2X_7R$). This receptor is a nondesensitizing plasma membrane ion channel that assembles from three or more 595 AA-long subunits (Surprenant *et al*., 1996; Kim *et al*., 2001). The amino-acid sequence predicts a membrane topology with two transmembrane stretches, a bulky extracellular domain and cytoplasmic N- and C-termini. In the extracellular region, there are 10 cysteines, three N-linked glycosylation sites and 18–21 lysines, depending on the species of origin. Upon transient stimulation, the $P2X_7R$ behaves like many other channels selective for mono and divalent cations. However, upon sustained stimulation the aqueous pore dilates to admit molecules with a molecular mass up to 900 Da, irrespective of the charge.

The physiological functions of the $P2X_7R$ are largely unknown, but researchers have been long intrigued by the potent cytotoxic effect caused by its sustained stimulation and by the ability of this receptor to drive the release of large amounts of IL-1β (and other inflammatory cytokines) from immune and inflammatory cells (Di Virgilio *et al*., 2001). Mice with the *P2x7* gene deleted ($p2x7^{-/-}$) have been generated and are currently being exploited to investigate $P2X_7R$ function. These animals are fertile and do not show dramatic phenotypic alterations, however, they appear to have impaired bone formation and remodelling, and reduced ability to develop acute inflammation of the joints in a typical model of experimental arthritis induced by anticollagen antibodies (Labasi *et al*., 2002; Ke *et al*., 2003). These *in vivo* observations strengthen previous *in vitro* data hinting at an important modulatory role of the $P2X_7R$ in immune cell physiology. However, acceptance of $P2X_7$ as an important immunomodulatory receptor, and, as a result, identification of this receptor as a promising target for the development of novel anti-inflammatory drugs, has been so far precluded by the frustrating lack of potent and selective blockers.

Two compounds bearing aldehyde functions, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and periodate-oxidized ATP (oATP), are currently in use as $P2X_7R$ blockers. PPADS was initially introduced as a wide-spectrum P2 inhibitor (Lambrecht *et al*., 1992), and later shown to be 10- to 20-fold selective for P2X over P2Y receptors. oATP was originally introduced as an affinity reagent for nucleotide-binding-proteins, and later proposed as a selective antagonist of macrophage $P2X_7R$, with no antagonist activity on coexpressed P2Y receptors (Murgia *et al*., 1993). Inhibition by oxidized ATP is irreversible and requires a prolonged (1–2 h) incubation. Later studies showed that that $P2X_1R$ and $P2X_2R$ are also blocked by this reagent.

The rationale for using oATP as a $P2X_7$ receptor antagonist was that the nucleotide structure of this Schiff-base-forming reagent might allow selective modification of lysine residues in the vicinity of the ATP-binding site of the $P2X_7R$, and thus block the receptor. Over the last 10 years, oATP has proven to be a valuable $P2X_7R$ reagent when used to antagonize ATP-dependent stimulation of this receptor, less valuable when used to infer the participation of $P2X_7R$ in cellular responses due to stimulation with non-nucleotide ligands. In this issue of the *British Journal of Pharmacology*, George Dubyak and co-workers (Beigi *et al*., 2003) have examined oATP effects in three human cell lines lacking $P2X_7R$ expression, and provide sound evidence in support of an inhibitory effect of this reagent independent of $P2X_7R$. This observation raises two

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important questions: (1) under which conditions can oATP be used as a reliable $P2X_7$ receptor blocker? (2) What are the site(s) and mechanism(s) of the $P2X_7R$-independent anti-inflammatory effect of oATP? As regards the conditions under which oATP can be used as an unambiguous probe of $P2X_7R$ function, Beigi *et al*. (2003) confirmed that blockade of ATP-induced Ca^{2+} influx, inward currents or membrane permeabilization by oATP in the vast majority of cases are due to $P2X_7R$ blockade. With respect to the mechanism of $P2X_7R$-independent actions of oATP, these authors suggest that it might directly interfere with cytokine or pattern recognition receptors (PRR), such as Toll-like receptors, expressed on the plasma membrane of immune cells. Alternatively, they hypothesize that oATP might gain access to the cell cytoplasm (maybe by fluid-phase pinocytosis), and covalently modify and inhibit intracellular kinases. However, I think that an alternative (or additional) pathways should also be considered.

oATP is a Schiff-base-forming reagent and an immunomodulatory action of Schiff-base-forming compounds is not new. Small Schiff-base-forming molecules have long been known to mimic cell surface ligands expressed by antigen-presenting cells and provide costimulatory signals to $CD4^+$ T cells, with a resulting Th1 polarization of the T cells (Rhodes *et al*., 1995). One such compound, tucaresol, developed at Glaxo Smith Kline, is currently being evaluated as an oral immunopotentiatory drug to be associated with vaccines. *In vitro* and *in vivo* studies show that Schiff-base formation by tucaresol increases T-cell receptor-dependent cytokine production and the ability to cope with tumors and with viral, bacterial and protozoal infections (Charo *et al*., 2002). The mechanism of this effect of tucaresol is unclear and the plasma membrane receptors involved have never been identified. Furthermore, the two compounds tucaresol and oATP have opposite effects on immune cell responses: stimulatory effects for the former, and inhibitory effects for the latter. However, this is not a major problem, as the effect might be dose-dependent, and a proper analysis of the dependency of oATP effects on the dose has not yet been carried out. Quite interestingly, Schiff-base-forming drugs seem to mimic the effect of endogenous Schiff-base-forming compounds, such as *p*-hydroxyphenylacetaldehyde, a small highly reactive molecule produced by activated neutrophil myeloperoxidase. This neutrophil metabolite is also a potent amplifier of Th1 responses *in vitro* and *in vivo*. As suggested by Rhodes (2002), small Schiff-base-forming products, endogenously generated in inflammatory conditions, might function as danger signals linking the innate and adaptive immune responses. It is intriguing to notice this additional convergence between Schiff-base-forming compounds and P2R, since extracellular nucleotides are currently viewed as early danger signals at inflammatory sites (Gallucci & Matzinger, 2001), and P2R as sensors of danger (La Sala *et al*., 2003).

In conclusion, according to the new data provided by Beigi *et al*. (2003), there are few doubts that oATP inhibits more cell receptors and pathways than just the $P2X_7$ type, but this is not by itself surprising: many so-called 'selective' receptor antagonists have turned out to be much less selective than initially thought. What emerges from these studies is that oATP can be used as a reliable tool to study $P2X_7$ receptor functions only under selected experimental conditions, in particular when the stimulant is a $P2X_7R$ agonist such as ATP or benzoylbenzoyl ATP (BzATP), the read-out is a *bona fide* $P2X_7R$-dependent response, and an independent control, for example, $P2X_7R$-less clones of the same lineage as the cells under investigation, is available. Outside these boundaries, oATP inhibition should not be taken as unequivocal indication of the involvement of $P2X_7R$, and in any case the results should be interpreted with extreme caution. Does this mean the end of oATP as a tool to investigate purinergic modulation of immune cells? Probably not. Recognition of effects of oATP unrelated to $P2X_7R$ blockade might shed light on the long-known immunomodulatory effect of Schiff-base-forming compounds, help to understand better the mechanisms involved in immunostimulation, and suggest new approaches for the development of immunomodulatory drugs.

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(Received July 9, 2003
Revised July 21, 2003
Accepted July 22, 2003)