Signalling by extracellular nucleotides in health and disease☆

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ABSTRACT

Nucleotides are released from all cells through regulated pathways or as a result of plasma membrane damage or cell death. Outside the cell, nucleotides act as signalling molecules triggering multiple responses via specific plasma membrane receptors of the P2 family. In the nervous system, purinergic signalling has a key function in neurotransmission. Outside the nervous system, purinergic signalling is one of the major modulators of basal tissue homeostasis, while its dysregulation contributes to the pathogenesis of various disease, including inflammation and cancer. Pre-clinical and clinical evidence shows that selective P2 agonists or antagonists are effective treatments for many pathologies, thus highlighting the relevance of extracellular nucleotides and P2 receptors as therapeutic targets.

1. Introduction

Nucleotides (mainly ATP, ADP, UTP, UDP, and UDP-glucose) are ubiquitous molecules involved in the control of a multiplicity of cellular processes, acting as coenzymes, energy intermediates, allosteric modulators, hydrotropes, as well as intracellular and extracellular messengers. The first evidence that nucleotides participate in cell-to-cell communication as intercellular messengers was provided in the peripheral nervous system by Geoffrey Burnstock, who first coined the term "purinergic signalling" to describe this novel type of intercellular communication [1]. Despite widespread initial scepticism, it is now clear that extracellular nucleotides, notably ATP, are ubiquitous signalling molecules, mainly released during neurotransmission, but also in response to a variety of cell stressors such as mechanical distortion, osmotic swelling, hypoxia, and cytotoxic agents. Thus, nucleotides may be released following cell injury or via non-lytic pathways such as secretory exocytosis or plasma membrane channels [2]. Once in the extracellular space, nucleotides bind and activate receptors of the P2 family. Virtually, all mammalian cells express P2 receptors (P2Rs) with individual ligand specificity and widely different affinity, thus conferring to the purinergic system a remarkable plasticity allowing fine tuning of many pathophysiological responses [3].

2. Nucleotide release

ATP release is well documented in the nervous system, where this nucleotide serves as a fast neurotransmitter itself or as a modulator of the release of other neurotransmitters [4]. In nerve cells, ATP is stored in synaptic vesicles, and thus can be released by secretory exocytosis, although extrasynaptic release is also documented. Over the years, it has become clear that ATP release is very common and extends well beyond the nervous system. At sites of inflammation or tissue damage, injury of the plasma membrane allows passive efflux of vast amounts of nucleotides due to the large intracellular/extracellular nucleotide gradient. However, besides passive leakage, nucleotides may also be released via non-lytic mechanisms, such as stimulated or constitutive exocytosis, microvesicle shedding, connexin or pannexin hemichannels, maxi-anion channels (MACs), calcium homeostasis modulator 1 (CALHM1), membrane transporters (ATP-binding cassette, ABC, transporters), and members of the P2 receptor family such as the P2X7 receptor (P2X7R) [5]. Accurate measurement of the extracellular ATP (eATP) concentration is a debated and not yet fully settled issue. Seminal experiments by Forrester showed that in the venous effluent of exercising human arms a concentration of up to 1 μM eATP could be detected [6]. In vitro, bulk eATP concentration in the supernatant of airway smooth muscle cells exposed to mechanical stretch was estimated to be 10–30 nM [7], but of course this does not reflect the actual eATP concentration in the

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pericellular space, let alone the eATP concentration in the interstitial milieu in vivo. Osmotic swelling-induced ATP release reached a concentration of 5–20 μM in the vicinity of the cell surface of various cell types such as CFTR-transfected mouse C127 cells, cardiac myocytes and the human intestine cell line 407 [8]. Similar eATP concentration increases were reported in β pancreatic cells stimulated with glucose, macula densa cells stimulated with NaCl, and airway epithelial cells stimulated with dibutyryl-cAMP [8]. Local eATP concentration on the surface of rat lung alveolar cells exposed to mechanical stretch was estimated to be 4–5 μM [9]. In vivo tissue injury caused by microbubble cavitation has been reported to cause ATP release to a concentration higher than 250 μM, and a similar eATP concentration has been measured in xenografted tumors in mice [10]. These eATP levels are close to those determined by us in a few experimental cancer model systems [11,12]. Although these data suggest that different stimuli (e.g. osmotic stress vs physical injury or inflammation) cause different levels of ATP release, they provide little indication as to the pathway involved, except that in the case of injury, where ATP release is most likely due to plasma membrane damage.

Once in the extracellular space, nucleotides are degraded by different cell surface or soluble enzymes (ecto-nucleotidases) which hydrolyze nucleoside tri-, di-, and mono-phosphates to the respective nucleosides. There are four major families of ecto-nucleotidases: ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases) including CD39, CD39L and CD39L3, ecto-5′-nucleotidase (5'-NT, also known as CD73), ecto-nucleotide pyrophosphatases/phosphodiesterases (ENPPS) and alkaline phosphatases (APs) [13]. The ENTPDase CD39 is expressed by virtually all tissues, where it hydrolyzes ATP to ADP and AMP. AMP is further hydrolyzed to adenosine by 5'-NT/CD73, which is the major enzyme producing extracellular adenosine from AMP. CD73 can also hydrolyze CMP, UMP, IMP and GMP, but with lower affinity than AMP. Adenosine is further degraded to inosine by adenosine deaminase (ADA). ENTPDases also hydrolyze dinucleoside polyphosphates, ADP ribose and NAD$^+$, but with low affinity. CD39 and CD73 are probably the most important nucleotide-hydrolyzing enzymes involved in purinergic signalling. Their expression and function are stimulated by hypoxia and during inflammation, and are critically involved in immunomodulation in the tumor microenvironment (TME) [14,15].

3. Extracellular nucleotide detection

It is an established notion that eATP and other nucleotides are crucial constituents of the inflammatory milieu [16], thus highlighting the need to develop techniques for their accurate measurement. Many sophisticated approaches to measure the nucleotide concentration in the pericellular space have been proposed over the years: enzyme-coated microelectrode biosensors, hexokinase and glucose-6-phosphate dehydrogenase-driven tandem enzyme reactions, atomic force microscopy, patch-clamped responder cells expressing an ATP-sensitive ion channel placed in the vicinity of the surface of the ATP-releasing cell (see Falzoni et al. for review [17]). More recently, additional techniques have been put forward, such as an enzymatic assay based on the quantification of extracellular UDP-galactose [18], a Forster resonance energy transfer (FRET)-based, plasma membrane-expressed sensor [19], a pH-sensitive, plasma membrane-targeted, FRET-based nano-machine [20], or a HPLC-based method for the simultaneous quantification of different nucleotides [21]. However, the more reliable and well established technique for ATP measurement is still the canonical, old-fashioned, luciferase that, in the presence of the co-factor luciferine, hydrolyzes ATP with the emission of photons (luminescence). Photon emission is proportional to the ATP concentration, thus measurement of light allows accurate determination of the ATP concentration [22].

The first technique to provide reliable measurements of the eATP concentration in vivo was based on the recombinant probe named pmeLUC (plasma membrane luciferase) [23]. This genetically-encoded probe exploits the canonical firefly luciferase engineered to be expressed on the plasma membrane, with the catalytic, ATP-binding, site facing the pericellular milieu. The pmeLUC probe can be expressed in virtually all cell types amenable to transfection, which can be then inoculated in vivo and used as sensors of the eATP concentration at different healthy or diseased sites. In alternative, pmeLUC-transfected tumor cells can be used to generate a primary tumor, thus allowing direct measure of eATP in the TME [22].

4. Nucleotide receptors

Extracellular nucleotides act via specific and widely distributed plasma membrane receptors: the P2 receptors. The P2 receptor family comprises the ionotropic P2X receptor (P2XRs, seven members) and the metabotropic P2Y receptor (P2YRs, eight members in humans) subfamilies (Fig. 1). P2XRs (P2X1R-P2X7R) are homo- or hetero-trimeric ATP-gated cation channels generated by the assembly of transmembrane subunits (from 384 aa for the shortest subunit, P2X4, to 595 aa for the longest, P2X7) with a large extracellular domain and N- and C-termini both on the cytoplasmic side. Genes encoding P2XRs have been identified in all vertebrates and lower eukaryotes, but not in prokaryotes. Basically, all P2XRs but the P2X7R can assemble with one another to form functional receptors, (i.e. P2X2/3, P2X1/5) [24,25]. The extracellular domain harbours 10 cysteine residues conserved in all P2XRs, and the ATP-binding sites (binding of 3 ATP molecules is requested for activation) localized at the site of contact of adjacent subunits. X-ray crystallography and cryoelectron microscopy shed light on the molecular structure of these receptors and allowed definition of the closed and open (ATP-bound) states as well as the identification of binding sites of inhibitors acting as negative allosteric modulators [26,27].

Extracellular ATP is the only known physiological agonist for all P2XRs. Opening of P2XRs allows large Ca^{2+} and Na$^+$ influx and K$^+$ efflux, thus leading to an increase in the intracellular Ca^{2+} concentration, cellular Na$^+$ overloading and K$^+$ depletion [28]. The disruption of the monovalent ion distribution causes membrane depolarization that may in turn trigger opening of voltage gated Ca^{2+} channels, thus promoting further Ca^{2+} influx. The increase in the cytoplasmic Ca^{2+} concentration activates a host of additional transduction pathways such as phospholipases, mitogen-activated protein kinases (MAPKs), and calmodulin-dependent enzymes. In addition, the large drop in intracellular K$^+$ caused by opening of the P2X7R subtype is a strong stimulus for the activation of the NLRP3 inflammasome, and therefore for the maturation and release of the potent pro-inflammatory cytokine IL-1β [29] (Fig. 2). Extracellular ATP has an ancillary role as fast neurotransmitter in the central nervous system, where it mainly acts as a modulator of the release of other neurotransmitters. Both glia and neurons express different P2XR subtypes, P2X1/P2X5R and P2X4R mainly on astrocytes, P2X7R on microglia and oligodendroglia, P2X2R in brainstem neurons. The P2X4R is also found on the postsynaptic membrane in CNS neurons where it is thought to mediate synaptic signalling and neuronal plasticity [30]. P2X2Rs and P2X3Rs are mainly found in peripheral nerve endings where they mediate sensory stimulation [31].

Among P2XRs, the P2X7R exhibits unique molecular structure and functional properties, such as the extended C-terminal tail (239 aa), typical of this receptor. Presence of this long cytoplasmic domain endows the P2X7R with the ability to form a large non-selective pore (macropore) permeable to water soluble molecules of MW up to 900 Da, nucleotides included [32,33]. At variance with other P2XRs (e.g. P2X1R and P2X3R), the P2X7R is slowly- or non-desensitizing with typical currents showing a steady increase, or facilitation, with repeated agonist applications [34]. The contribution of current facilitation to formation of the macropore is unclear since, despite previous claims that the P2X7R-associated macropore opens only after prolonged or repeated stimulation, it is now clear that large increases in conductance occur with no delay after P2X7R gating [35,36]. Recently, the full-length rat
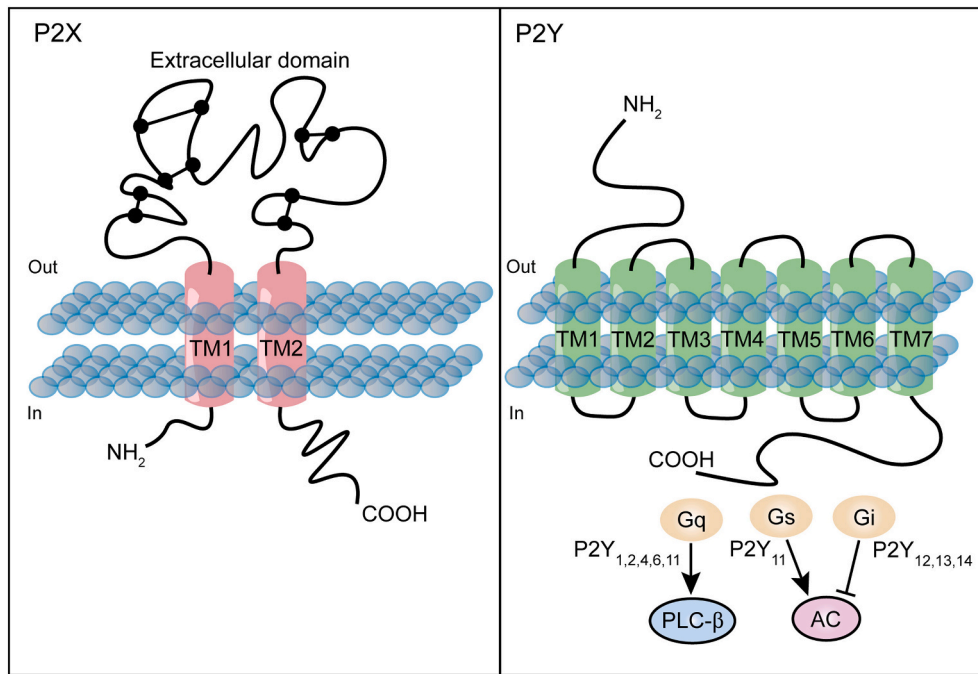
Fig. 1. Plasma membrane topology and coupling to intracellular messenger generation. The basic P2X subunit has both NH_2 and COOH termini on the cytoplasmic side of the plasma membrane. The outer domain shows ten cysteines (black dots) linked by disulfide bridges. P2YRs are typical G protein- coupled receptors possessing seven membrane-spanning domains with an extracellular NH_2 and an intracellular COOH domain. Coupling to different G proteins allows P2YRs to stimulate or inhibit different intracellular transduction systems (e.g. phospholipase C-β, PLC-β, or adenylyl cyclase, AC).
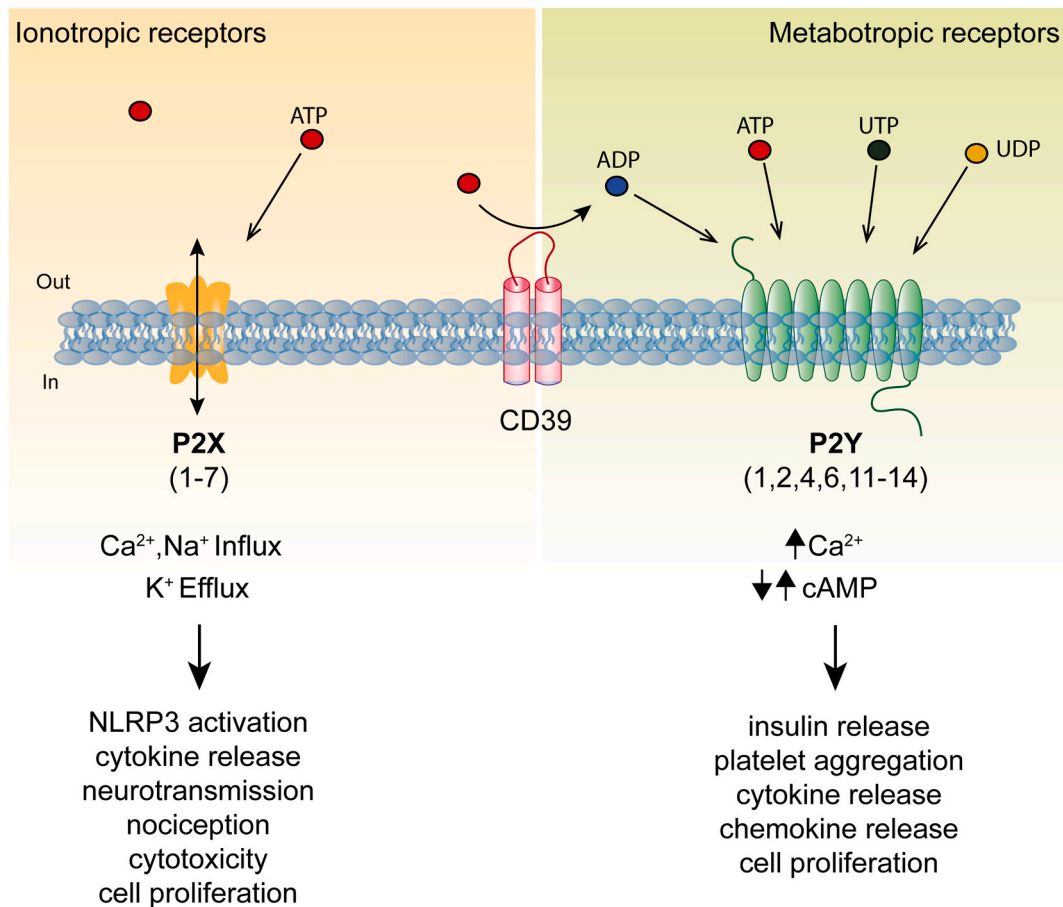

Fig. 2. P2XRs and P2YRs are mainly associated to the generation of fast (i.e. transmembrane ion fluxes)- or slow (i.e. Ca^{2+} release from intracellular stores or changes in the cAMP concentration)- acting intracellular messengers, respectively. However, the increase in the intracellular ion concentration caused by inotropic P2XRs may also drive slow cellular responses such as proliferation or cytokine release. Likewise, the intracellular Ca^{2+} increase caused by P2YR stimulation may cause fast responses such as neurotransmitter secretion. The only nucleotide active at P2XRs is ATP, while several nucleotides are active at P2YRs. CD39 has a pivotal role in purinergic signalling because it terminates P2XR stimulation by hydrolysing ATP and contextually generates ADP, a P2Y agonist.

receptor structure was resolved by cryoelectron microscopy, unveiling that the cytoplasmic C-terminal tail forms a peculiar "ballast-like" structure of unknown function [27]. More than fifty putatively P2X7R-interacting proteins have been identified, of which at least 20 implicated in immunity (e.g. MyD88 and NLRP3), but definition of the P2X7R "interactome" is still very preliminary and controversial (see [37] for a recent critical update). Among all P2Rs, the P2X7R has the lowest affinity for eATP, thus it has been proposed that in healthy tissues, where the eATP concentration is nanomolar, this receptor should be mostly silent, and be active only at inflammatory and tumors sites, where the eATP content can reach a concentration of hundreds of micromoles/L. However, accruing evidence suggests that the P2X7R is active and fulfils important trophic functions in immune cells (e.g. T lymphocytes or microglia) even at low eATP concentrations [38–40]. The reader is referred to a recently published review for further information on P2X7R structure and function [41]. While P2XRs are mostly implicated in neurotransmission, the P2X7R and P2X4R are mainly involved in signalling in inflammation and immunity. Intriguingly, the P2X4R besides the plasma membrane is also localized in the lysosomal compartment where it mediates lysosome fusion and Ca^{2+} release [42].

P2YRs are G-protein coupled metabotropic receptors comprised of eight members in humans (P2Y$_1$R, P2Y$_2$R, P2Y$_4$R, P2Y$_6$R, and P2Y$_{11}$R-P2Y$_{14}$R, missing numbers in the P2YR series refer to either receptors that are unresponsive to nucleotides or to non-mammalian orthologs; the *P2y$_{11}$* gene is absent in the rodent genome), and grouped into two subfamilies, depending on their sequence divergence and phylogeny [43,44]. This classification also reflects coupling to intracellular messenger generation. P2Y$_1$R, P2Y$_2$R, P2Y$_4$R and P2Y$_6$R activate G$_q$ and phospholipase C-β (PLC-β), generating the two intracellular second messengers inositol 1,4,5-triphosphate (IP3) and diacylglycerol, which increase intracellular Ca^{2+} and activate PKC, respectively. P2Y$_{11}$R activates both G$_q$ and G$_s$, thus triggering increases of both intracellular Ca^{2+} and cyclic adenosine monophosphate (cAMP). P2Y$_{12}$R, P2Y$_{13}$R and P2Y$_{14}$R activate G$_i$, thus inhibiting adenylyl cyclase (AC) and lowering the intracellular cAMP concentration [45]. Recently, additional intracellular signalling pathways activated by P2YRs have been described, including the recruitment of the G$_{βγ}$ subunit, leading to activation of a variety of effectors such as phosphatidylinositol-4,5-biphosphate 3-kinase γ (PI3K-γ), phospholipase C-β2 and −β3, G protein-coupled receptor (GPCR) kinase 2 and 3, Rho, and MAPKs [46]. These findings highlight the plasticity of nucleotide-stimulated intracellular signalling. Several different nucleotides are agonists at the P2YRs. ADP is the preferred ligand at P2Y$_1$R, P2Y$_{12}$R and P2Y$_{13}$R; UTP at P2Y$_4$R, while UDP at P2Y$_6$R. The P2Y$_{14}$R is predominantly activated by UDP, UDP-glucose or UDP-galactose. Both UTP and ATP are agonists at the P2Y$_2$R, while P2Y$_{11}$R is the only truly ATP-selective receptor at low ATP concentrations. High concentrations of ATP can also activate P2Y$_1$R and P2Y$_{13}$R, and inhibit human P2Y$_4$R and P2Y$_{12}$R [45]. Like the P2XRs, P2YRs are ubiquitously distributed and expressed by virtually all cell types, whether healthy or malignant, participating in a wealth of cellular responses, from proliferation to cell death, from motility to differentiation, from cytokine secretion to hormone release.

Purinergic signalling also includes adenosine and its cognate G protein-coupled P1 receptors: A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$. Activation of P1 receptors triggers AC stimulation (A$_{2A}$, A$_{2B}$) or inhibition (A$_1$, A$_3$), thus increasing or decreasing intracellular cAMP concentration, respectively. Therefore, the purinergic system is an integrated signalling network where the primary messenger (ATP) ligates a large family of receptors (P2YRs and P2XRs), drives multiple cellular functions, is degraded by extracellular enzymes (ecto-nucleotidases), and finally generates an additional mediator (adenosine) that binds another, smaller, family of receptors (A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$) that in turn drive additional, and often opposite, responses.

5. Signalling by extracellular nucleotides as therapeutic target

Extracellular nucleotides and P2Rs have become a field of fertile investigation and are considered promising pharmacological targets, but only in a very few cases these efforts have brought new drugs to the patient's bed [3]. Nevertheless, some robust clinical applications have been implemented over the years, chiefly in the cardiovascular system (Table 1).

Table 1

Receptor	Agonist	Antagonist	Current clinical applications	Potential clinical indications
P2Y$_2$	Diquafosol Denufosol		Dry eye disease	
				Cystic fibrosis
P2Y$_6$		TIM-38, MRS2578		Cardiovascular diseases, obesity
P2Y$_{12}$		Prasugrel, Clopidogrel, Cangrelor, Ticagrelor	Acute coronary syndrome, coronary artery disease, atherothrombotic events	
P2X3		AF-353		Hypertension, atherosclerosis
		AF-219 (Gefapixant)	Chronic cough	
		DT-111		Chronic obstructive lung disease, chronic cough
		BLU-5937		Cough
P2X4		NC-2600	Non-productive cough	Neuropathic pain
		PSB15417, NP-1815-PX		Neuropathic pain
P2X7		AZD9056		Hypertension, atherosclerosis, Crohn's disease
		AZ10606120, A740003, DHTS		Retinal diseases
				Early stage of tauopathy
		GSK1482160		Depressive disorder
		JNJ54175446		Rheumatoid arthritis
		CE224535		Lung tumor
	HEI3090 (positive allosteric modulator)			
nfP2X7		Antibody		Basal cell carcinoma (BCC)

5.1. Thrombosis and cardiovascular diseases

The most important patho-physiological setting in which a role for purinergic transmission is established beyond any possible doubt, with relevant clinical applications, is blood coagulation. It has been known for decades that ADP is a potent stimulus for platelet aggregation, well before the platelet cognate receptor, $P2Y_{12}R$, was pharmacologically characterized and cloned. A potent family of $P2Y_{12}R$ antagonists with a strong inhibitory activity on platelet aggregation, the thienopyridines, was developed at Sanofi and has entered medical practice as anti-aggregants and anti-thrombotic agents [47]. Thienopyridines (ticlopidine) were first approved for medical use in 1978. New generation thienopyridines (prasugrel, clopidogrel, cangrelor) or the cyclopentyltriazolo-pyrimidine ticagrelor are currently used for the treatment of acute coronary syndrome, coronary artery disease and atherothrombotic events [48]. Experimental evidence shows that the $P2Y_1R$ might also be a target for anti-platelet therapy [49], but so far this did not lead to the introduction of anti-thrombotic agents based on its antagonism.

Targeting $P2Y_4R$, $P2Y_6R$, $P2Y_{11}R$, $P2Y_{12}R$, P2X3R, P2X4R or P2X7R has been explored for the therapy of heart diseases, including infarction, arrhythmias, cardiomyopathies and angina [50]. The P2X3R antagonist AF-353 has been investigated for the treatment of hypertension and atherosclerosis as well as the P2X7R antagonist AZD9056 [51]. Some $P2Y_6R$ antagonists have been evaluated as potential drugs for the treatment of cardiovascular diseases, e.g. TIM-38 or MRS2578, this latter being the most potent $P2Y_6R$ inhibitor so far available [52].

5.2. Eye diseases

Eye diseases are an active field of P2 research. A $P2Y_2R$ agonist (Diquafosol, Santen Pharmaceutical, Japan) is currently used in clinical practice in Far Eastern Countries for the treatment of dry eye [53]. Recently it has been proposed that P2X7R might also play a role in the onset of this disease since P2X7R activation causes goblet cell death, which is an hallmark of irreversible chronic dry eye [54]. Compelling pre-clinical evidence shows that P2X7R over-activation promotes retinal damage associated to multiple diseases, e.g. diabetic retinopathy and age-related macular degeneration (AMD), and accordingly P2X7R inhibition counteracts inflammation in several in vivo and in vitro models of retinopathy [55], preventing retinal damage due to increased ocular pressure, and reverting hyperglycemia-dependent retinal injury [56]. Besides the widely used P2X7R antagonists, such as AZ10606120, A740003 or JNJ-5417544641, Bucolo and coworkers recently suggested that a natural diterpenoid (dihydrotanshinone, DHTS) might also inhibit P2X7R activity and prevent blood-retinal barrier breakdown, which is an hallmark of diabetic retinopathy [57].

5.3. Neurodegenerative and psychiatric diseases

Accruing evidence shows that P2Rs play an important role in a variety of neurological disorders, such as multiple sclerosis (MS), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). Recent data have rekindled interest in $P2Y_1R$ and P2X7R as possible players in the pathogenesis of AD [58,59]. The P2X7R antagonist GSK1482160 has been proposed for the treatment of early stage tauopathy [60], and a possible re-purposing of the widely used di-hydropyridine drugs has also been suggested [61]. $P2Y_6R$ and P2X7R have been implicated in ALS, but data are controversial and whether inhibition or stimulation of the P2X7R is beneficial is unclear [62]. In a recent study P2X7R/NLRP3 inflammasome activation has been linked to the development of Parkinson's disease (PD) [63], suggesting that P2X7R antagonists might be explored for treatment-naïve PD individuals. Mood disorders are probably the most promising field of application of P2X7R-targeting drugs so far. Convincing pre-clinical data, supported by genetic association studies [64,65] (but see [66]) show that P2X7R blockade is very

efficacious in animal models of neuroinflammation and anhedonia, pointing to a clinical application for the treatment of depression [67]. The P2X7R antagonist JNJ-54175446 is currently in Phase 2 clinical trial for treatment of depressive disorder (clinicaltrial.gov, NCT04116606). Other CNS diseases that might benefit from P2X7R inhibition are autism [68] and epilepsy [69].

5.4. Neuropathic pain

Extracellular ATP and other nucleotides are also potent regulators of neurons and glial cells in the pain pathway. In recent decades, an accumulating body of literature has provided evidence for the crucial role of the P2X3R, P2X2/3R, P2X4R and P2X7R in neuropathic pain [70]. $P2Y_1R$, $P2Y_2R$, $P2Y_6R$, $P2Y_{12}R$, and $P2Y_{13}R$ have been also implicated, based on pre-clinical evidence that inhibition of any of these receptors attenuated neuropathic pain triggered by diverse disease conditions [71], but despite several P2YRs inhibitors are available, no clinical trials on neuropathic pain are reported. As regard P2X4R, the novel potent antagonists NC-2600, active at both rodent and human receptors, was successfully tested in a phase I study by the Japanese Drug Company Nippon Chemiphar [72]. Other recently developed potent P2X4R antagonists are NP-1815-PX and PSB-15417. However, to our knowledge, there were no further developments on NC-2600 for the treatment of neuropathic pain, while in the summer of 2021 Nippon Chemiphar issued a press release launching NC-2600 as a prescription drug for the treatment of non-productive cough (https://www.biospace.com/article/nippon-chemiphar-presents-promising-data-on-novel-prescription-cough-medicine-representing-possibly-second-new-rx-cough-drug-to-enter-market-since-1950s/).

5.5. Bronchial and lung diseases

Purinergic signalling is being actively explored for the treatment of airway diseases. Increased ATP levels are found in the airways of asthmatic patients or mice with experimentally-induced asthma where multiple P2R subtypes are thought to be involved, including $P2Y_1R$, $P2Y_6R$, P2X1R and P2X7R [73]. The P2X3R selective antagonist AF-219 (now MK-7624, commercial Gefapixant) has been approved by the FDA in 2021 for treatment of refractory or unexplained chronic cough. Recently a chemically-unrelated P2X2/3R water soluble antagonist (DT-111) was shown to inhibit the neurogenic reflex in chronic obstructive lung disease (COPD) and to alleviate chronic cough [74]. An anti-tussive effect was also reported for additional compounds, e.g. BLU-5937, eliapixant and sivopixant, with potent and selective P2X3-blocking activity (https://www.ajmc.com/view/companies-provide-trial-updates-on-p2x3-blockers-for-chronic-cough). The therapeutic potential of $P2Y_2R$, $P2Y_4R$, $P2Y_{11}R$ and P2X4R has also been explored for the treatment of cystic fibrosis, and a potent and hydrolysis-resistant inhalatory $P2Y_2R$ agonist, (Denufosol), was taken to Phase III clinical development [75]. However, in the second Phase III study it did not meet the primary endpoint compared to placebo, thus clinical experimentation was halted. Better hopes for the treatment of non-productive cough seem to rest on the use of the P2X4R blocker NC-2600, as reported above.

A number of unrelated conditions (sepsis, shock, inhaled toxics, viral infections among which Covid-19) can trigger a devastating acute lung inflammation referred to as Acute Respiratory Distress Syndrome (ARDS). No effective treatments are so far available. An experimental model that replicates many of the features of human ARDS is ALI, acute lung injury, caused by the inhalation of bacterial lipopolysaccharide. A pathogenic role for eATP and the P2X7R has been identified in this severe lung inflammation, thus advocating the therapeutic use of P2X7R antagonists [76].

5.6. Chronic inflammatory diseases

P2X7R and other P2YRs, such as $P2Y_1R$, $P2Y_2R$ are also implicated in

the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE) [77]. In SLE there is evidence that reduced expression of the P2X7R by T follicular helper cells is permissive for the generation of self-reactive antibodies, and thus for host tissue damage [78].The P2X7R has been also extensively investigated as a therapeutic target for the treatment of rheumatoid arthritis, osteoarthritis, and Crohn's disease [79]. Encouraging results were obtained in phase II clinical trials for the treatment of Crohn's disease and rheumatoid arthritis with the P2X7R antagonists AZD9056 and CE224535 respectively [80,81], but not such to justify further clinical development.

5.7. Metabolic diseases

Emerging evidence supports the participation of extracellular nucleotides and P2 signalling in the pathogenesis of obesity-related inflammation. The P2X7R is up-regulated in the adipose tissue of individuals with metabolic syndrome. Deficiency of P2X7R attenuates inflammation, oxidative stress and liver fibrosis induced by high-fat diet (HFD) [82]. P2Y receptors are also involved in metabolic disturbances. P2Y6R antagonists, such as MRS2578, are promising anti-obesity agents since UDP, acting via P2Y6R in the hypothalamus, increases fat accumulation [83]. A recent study showed that *P2ry2*-knockout mice fed with an HFD display lower levels of hyperinsulinaemia and better management of blood glucose levels compared with wild-type HFD-fed mice [84]. It has been proposed that in HFD-fed mice increased pannexin-1-mediated eATP release from muscle fibres triggers P2Y2R overactivation, thus promoting inflammation and insulin resistance in skeletal muscle cells [85]. Furthermore, the observation that hyperglycemia drives enhanced eATP release by human and murine islets of Langerhans, and that P2X7R blockade delays diabetes onset in NOD mice and prevents hyperglycemic microvascular complications, points to the P2X7R as an attractive target for the treatment of diabetes [86].

5.8. Infection

The P2R most convincingly involved in inflammation and immunity is the P2X7R. This receptor, expressed by virtually all cells of innate and adaptive immunity, has a fundamental role in the control of bacterial (e.g. *Myobacterium tuberculosis, Porphiromomas gingivalis*), viral (e.g. HIV, Dengue virus-2) and parasitic (e.g. *Leishmania amazonensis, Trichomonas vaginalis*) infections [87,88]. Notably, there has been long-standing interest in the possible participation of the P2X7R in host defense against *Mycobacterium tuberculosis* based on the well documented potentiation of macrophage-mediated intracellular Mycobacterium killing following P2X7R stimulation [89]. However enthusiasm was mitigated by the difficulty to tune P2X7R activation to the level sufficient to promote intracellular Mycobacterium killing without causing at the same time extensive host cell death and tissue damage, due to the well-known P2X7R-dependent cytotoxicity. Recent experiments suggest a way around this problem since graded/controlled activation can be safely achieved at inflammatory sites with the administration of positive P2X7R allosteric modulators (e.g. clemastine). This approach exploits the increased eATP concentration typical of inflamed tissues, that permit efficient and, more importantly, selective P2X7R gating by the allosteric modulators. This strategy has been shown to be an effective therapy in a zebra fish model of tuberculosis [90].

Emerging evidence also suggests a role in infection for P2Y6R and P2Y2R. Bone marrow macrophages deficient of P2Y6R are susceptible to viral infections, and accordingly P2Y6R stimulation is protective [91]. On the other hand, depletion of P2Y2R reduces macrophages and lymphocytes accumulation and P2Y2R activation protects against viruses by supporting Th1 responses [92]. P2Y11R targeting has been shown to inhibit TNFα release [93], thus its stimulation might prevent deleterious effects of hyper- inflammation, as in Coronavirus disease 2019 (COVID-19). In this regard, the P2X7R might be an additional useful target as its blockade inhibits the release of multiple factors responsible of the thrombo-inflammatory syndrome typical of Covid-19 [94]. A role for the P2Y2R in facilitating HIV infection of CD4$^+$ cells has also been suggested [95]. Participation of the P2X7R in sepsis might be widespread due to its activation by the antibacter peptide LL37 released from activated neutrophils [96], and to the serious metabolic derangement and NLRP3 dysfunction caused by its overstimulation [97]. Besides the P2X7R, the P2X4R [98] and the P2Y12R have also been implicated in sepsis whether with a causative or protective function [99].

5.9. Cancer

Cancer is a very active field of investigation in the P2R field. Tumor interstitium, the TME, contains eATP at a concentration of hundreds micromoles/L [12]. Such a high eATP concentration profoundly modulates tumor/host interactions and can be exploited to confer selectivity to anti-cancer therapy [100–103]. The TME is the site of a complex bidirectional interaction between host and tumor cells since both partners release ATP (which contributes to establishing the high eATP level typical of this compartment), and both express a vast array of P2Rs, mainly P2X5R, P2X7R, P2Y1R, P2Y2R and P2Y11R. Thus, the net effect of activation of the purinergic system depends on a delicate balance between the tumor-suppressing and tumor-enhancing activity [104]. Stimulation of P2Y2R or P2X7R has a growth-promoting effect on cancer cells, thus driving tumor progression. But at the same time, P2Y2R and P2X7R stimulation causes immune cell recruitment and activation, thus driving tumor regression. Curiously, bilirubin has been recently identified as a novel P2X7R antagonist with an anti-tumor activity [105].

There is evidence that P2X7R activation by positive allosteric drugs may enhance anti-tumor immunity, and in combination with anti-PD1 administration achieve complete tumor eradication in mice inoculated with Lewis lung carcinoma cells [102]. The anti-tumor effect depends on the controlled stimulation of dendritic cell P2X7R, leading to release of IL-18 that in turn supports the effector functions of tumor-infiltrating NK cells [102]. On the other hand, administration of various P2X7R small molecule blockers was shown to slow down progression of many experimental tumors [106]. Despite increasing pre-clinical evidence, very few clinical studies in cancer patients have been so far performed. A phase I clinical trial showed that local administration of ointment containing an antibody raised against a non-functional P2X7R variant (nfP2X7) caused regression of basal cell carcinoma [107] (Clinicaltrial.gov NCT02587819). The P2X7R is also under investigation as a possible inflammatory marker in peritoneal carcinomatosis in women with ovarian or colonic cancer (Clinicaltrial.gov NCT04122937). Whether presence of specific single nucleotide polymorphisms (SNPs) in this receptor might influence anti-tumor immune response during neoadjuvant chemotherapy is also under investigation.

Activity of ecto-nucleotidases expressed by both immune cells and tumor cells in the TME is a complicating factor in the interpretation of the role played by P2 receptors in cancer. In the presence of ecto-nucleotidases (e.g. membrane-bound CD39, CD39L, CD39L3, or CD73, or soluble NTPDases 5 and 6 and alkaline phosphatase) eATP is hydrolysed to adenosine, which is then degraded to inosine by adenosine deaminase (ADA). Adenosine is a potent immunosuppressant, thus degradation of eATP in principle should depress the P2-mediated anti-tumor response and on the one other potentiate immunosuppression and thus facilitate tumor progression [108]. Several Phase 1 clinical trials (13 as of February 2022) listed on ClinicalTrials.gov are currently ongoing to test the safety and efficacy of CD39-targeting in human tumors alone or in association with immune check-point blockers (e.g. NCT04336098, NCT05075564, NCT04306900; NCT03884556). An even larger number of studies (38 as of February 2022) is currently investigating the safety and efficacy in cancer of CD73 blockade, as a stand-alone treatment or in combination with other drugs (e.g. NCT04148937, NCT04797468, NCT04572152, NCT05143970).

5.10. Acupuncture

Acupuncture is a therapeutic procedure of traditional Chinese medicine showing good results in the attenuation of chronic pain. Extracellular ATP locally released by acupuncture needling might contribute to the analgesic effect due to secretion of opioid peptides [109]. Scattered experimental evidence hints to a possible involvement of P2X3R, P2X4R, P2X7R, P2Y$_1$R and P2Y$_{13}$R in the analgesic effect of this procedure, although the eATP degradation product adenosine is also likely to be implicated [110].

6. Conclusion

Nucleotides, chiefly ATP, are ubiquitous components of the extracellular milieu. In healthy tissues, their concentration is very low, in the nanomolar range, although there is evidence that local concentration, for example at the neuronal synapses, can be substantially higher. On the contrary, it is now well established that in diseased tissues the eATP concentration is increased several fold to reach tens or even hundreds of micromoles/L. It is absolutely clear that signalling by eATP affects a multiplicity of cell functions, in health and disease, but we are still unable to harness the opportunities afforded by the increasing knowledge of this intercellular communication system. A big leap forward was made possible by the availability of reliable probes for the measurement of the eATP concentration in vivo: what had been simply hypothesized on the basis of indirect functional evidence can now be semi-quantitatively demonstrated [12,111]. Availability of several models of genetically-modified animals and the resolution of the molecular structure of P2Rs have provided additional fundamental information for drug design and mechanistic interpretation [27,112,113]. Thus, we can safely conclude that the role of purinergic signalling in pathophysiology is well established. Yet, with the exception of thrombosis, there is no major human disease in which drugs targeting purinergic signalling are a primary indication. This is the main challenge that purinergic students will be confronted with in the near future.

CRediT authorship contribution statement

All authors contributed to writing and revision of the review.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesco Di Virgilio reports a relationship with Axxam SpA that includes: consulting or advisory.

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