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ScienceDirect
Pharmacology & Therapeutics 112 (2006) 264 – 280
Pharmacology & Therapeutics
www.elsevier.com/locate/pharmthera

P2 receptor web: Complexity and fine-tuning

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Abstract

The present review offers a new perspective on a family of receptors, termed P2 receptors, specific for nucleoside tri- and diphosphates of purines/pyrimidines. We emphasize here that while decoding the inputs of various related extracellular ligands, P2 receptors are a clear example of increasing biological complexity. They are represented by 7 ionotropic P2X and 8 metabotropic P2Y receptors; they have very heterogeneous ligands and binding characteristics, molecular properties, transduction mechanisms, cellular localization and protein–protein interactions. While the reason for this sophistication is unknown, a few compelling issues emerge while looking at such a rich variety. We ask, for instance, why so many different receptor subtypes are necessary for triggering biological properties and functions, and if these receptors are more than the sum of their single entities. A first possibility is that newly synthesized P2 proteins are casually located on the cell surface (stochastic hypothesis). Alternatively, distinct subunits are engaged on different cell phenotypes by genetic control (genetic determinism) and/or selective recruitment under physiopathological conditions and epigenetic stimuli (epigenetic determinism). Nevertheless, an appropriate way to both dissect the vast biological scenario and molecular complexity among P2 receptors and to integrate and upgrade their assortment is to regard them as a "combinatorial receptor web", that is, a dynamic architecture of P2 proteins demonstrating economic efficiency and involving a process of "fine-tuning", a mechanism which endorses the dynamic nature of all biological reactions. In the present analysis, we stimulate a scientific query about what contributes to such a vast P2 receptor sophistication.

Keywords: Ionotropic P2X receptor; Metabotropic P2Y receptor; Receptor cross talk; Receptor web; Combinatorial calculation

Abbreviations: BzATP, 3′-*O*-(benzoyl-4-benzoyl)-ATP; CNS, central nervous system; DRG, dorsal root ganglia; GPCR, G protein-coupled receptors; GABA, γ-amino butyric acid; 5-HT, 5-hydroxytryptamine; IP_3, inositol triphosphate; α,β-meATP, α,β-methylene ATP; β,γ-meATP, β,γ-methylene ATP; NA, noradrenaline; PKC, protein kinase C; PLC, phospholipase C; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium; suramin, (8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid; TM, transmembrane.

Contents

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doi:10.1016/j.pharmthera.2005.04.012

1. Introduction

Extracellular nucleoside tri- and diphosphates, first of which the prototype ATP, are considered as the phylogenetically most ancient epigenetic factors sustaining a broad range of short-term and long-term biological effects. In several different tissues, these can vary from neurotransmission, smooth and cardiac muscle contraction, chemosensory signaling, secretion, vasodilatation, microglia activation, to more complex phenomena such as immune responses, male reproduction, fertilization and embryonic development (Burnstock & Knight, 2004; Illes & Ribeiro, 2004; Vial et al., 2004a; Burnstock, 2006a, 2006b, 2006c, 2006d). Effects as different as proliferation, differentiation, chemotaxis, release of cytokines or lysosomal constituents, generation of reactive oxygen or nitrogen species, are moreover elicited by extracellular ATP upon stimulation of blood cells. In addition to these well-described physiological activities, extracellular nucleotides are also recognized as having increasing importance in pathological conditions (Burnstock, 2004a; Gallagher, 2004; Cattaneo, 2005; Kennedy, 2005; Burnstock, 2006b). They have key roles in cancer, cardiopulmonary insufficiency, thrombosis, diabetes, skin and bone diseases, gut motility disorders, diseases of the ear and eye, bladder incontinence, behavioral disorders and pain (James & Butt, 2002; D'Ambrosi et al., 2004; Di Virgilio et al., 2005; White et al., 2005; Burnstock, 2006b). Particularly in the central nervous system (CNS), in addition to their established functions as neurotransmitters, cotransmitters, neuromodulators and growth factors (Burnstock, 2004b), extracellular nucleotides have recently been shown to have additional biological tasks ranging from survival, repair, remodeling during development, to involvement in injury, metabolism impairment, excitotoxicity, acute and chronic neurodegenerative conditions (Volonté et al., 2003; Franke & Illes, 2006). They participate in neuronal mechanisms triggered by axotomy in rat precerebellar nuclei (Florenzano et al., 2002), in astrocytic effects induced by stab wounds (Franke et al., 2004a), in reactive gliosis occurring after traumatic brain injury (Neary et al., 2005), in neuronal and glial responses to cerebral ischemia in vitro and in vivo (Cavaliere et al., 2002, 2004a; Franke et al., 2004b; Melani et al., in press) and in neuronal recovery after growth factor withdrawal (D'Ambrosi et al., 2000, 2001). It is well known that metabolic stress, brain ischemia and trauma often evoke massive extracellular release of ATP and additional excitotoxic neurotransmitters (Phillis et al., 1993; Juranyi et al., 1999; Melani et al., 2005), and extracellular ATP per se is noxious to primary CNS neurons (Amadio et al., 2002, 2005). Also it mediates hypoxic/hypoglycemic signaling in vitro (Cavaliere et al., 2001a, 2001b, 2002, 2004a, 2004b) and in vivo (Prasad et al., 2001; Cavaliere et al., 2003; Franke et al., 2004b). Consistent with this, several purinergic antagonists abolish the cell death fate of primary neurons exposed to excessive glutamate (Volonté & Merlo, 1996), serum/potassium deprivation (Volonté et al., 1999), hypoglycemia and chemical hypoxia (Cavaliere et al., 2001a, 2001b, 2002, 2004a, 2004b). Nevertheless, ATP released from cells (Neary et al., 1996; Bodin & Burnstock, 2001) acts not only on neurons, but also mediates microglial inflammatory processes involved either in pathological conditions, or in the protection of the CNS (Kreutzberg, 1996; Minghetti et al., 1999). For instance, ATP is a trigger for tumour necrosis factor-α secretion and a modulator for interleukin-1β release from cultured microglia, suggesting that outflow of ATP during degenerative events could also boost the pro-inflammatory response of already activated microglial cells (Di Virgilio et al., 1998, 2001). All these functions substantiate the high level of complexity of purinergic mechanisms.

2. Molecular and pharmacological classification

As expected in sustaining so many sophisticated biological tasks, the specific extracellular receptors for nucleoside tri/diphosphates, the P2 receptors, show heterogeneity at the molecular level. They are subdivided into ionotropic ATP-gated ion channels (P2X receptors), mediating rapid and selective permeability to Na^+, K^+ and Ca^{2+} (North, 2002) and into G protein-coupled metabotropic subtypes (P2Y receptors), inducing a slower onset of responses and involving second-messenger systems (Abbracchio et al., in press). Furthermore they are subdivided into 7 distinct P2X receptors ($P2X_{1-7}$) and 8 different P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) receptors cloned to date from mammalian species.

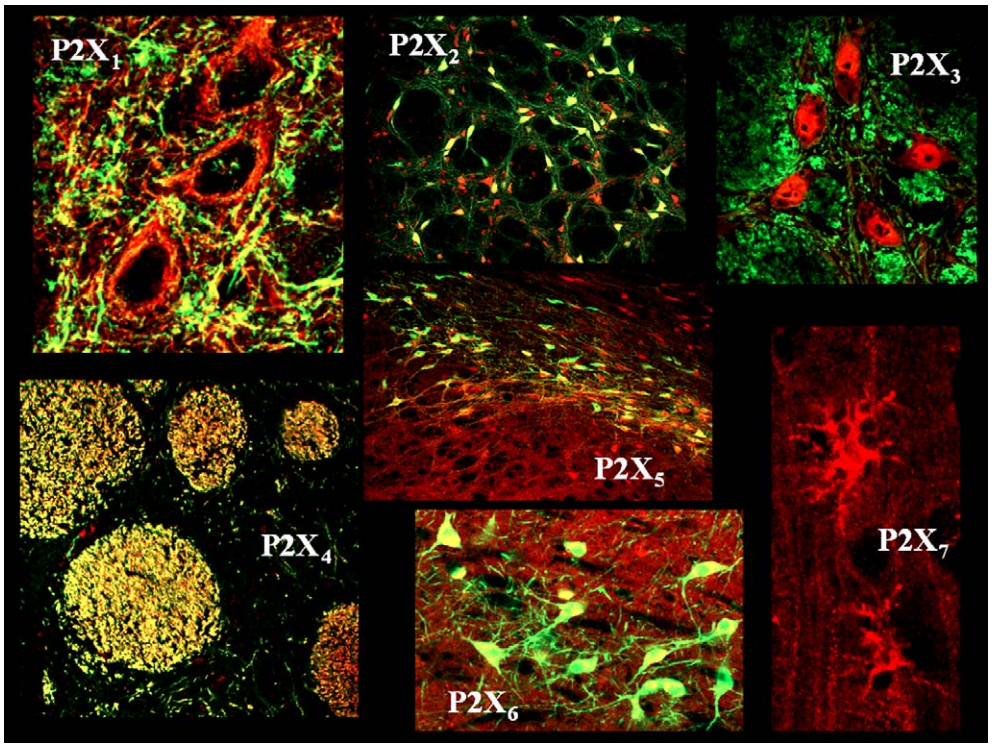

Fig. 1. P2X receptors are differently expressed in the CNS of adult rat. $P2X_1$ receptor is shown in cortical neurons (red); $P2X_2$ receptor is present in striatum (red and yellow); $P2X_3$ receptor is expressed in striatal neurons (red); $P2X_4$ receptor is observed in white matter in striatum (yellow); $P2X_5$ and $P2X_6$ receptors are found on neurons in substantia nigra pars compacta and reticulata, respectively (yellow); $P2X_7$ receptor is localized on microglia in cerebral cortex (red).

2.1. P2X receptors

P2X receptors (Fig. 1) define the third major family of ionotropic receptors (together with Cys-loop channels and glutamate-gated channels) and are present as 379–595 amino acid proteins, possessing tertiary structures with 2 transmembrane (TM) domains, intracellular N- and C-terminus, post-translational modifications including glycosylation and phosphorylation. Most of the conserved regions are in the extracellular loop, with the TM domains being less well conserved. The extracellular loop of cloned $P2X_{1-7}$ receptors has 10 conserved cysteine residues, 14 conserved glycine residues and 2–6 potential N-linked glycosylation sites. It is believed that disulfide bridges in the extracellular loop may form the structural constraints needed to couple the ATP-binding site to the ion pore. Histidine residues can mediate pH regulation of P2X receptors and form metal ion-binding sites (Clyne et al., 2002). P2X receptors can be regulated allosterically by extracellular protons, divalent cations and a range of metals (Brown et al., 2002); however, their effects are variable between receptor subtypes, as shown from mutagenesis experiments. Desensitization occurs for all P2X receptors, but the time scale appears to be very important, since in some P2X subtypes this decline lasts for milliseconds (fast desensitization: $P2X_1$, $P2X_3$ receptors), while in others it occurs 100 to 1000 times more slowly (slow desensitization: $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors) (Ralevic & Burnstock, 1998; North, 2002). Alternative splicing of P2X receptor pre-messenger RNA has been shown for the $P2X_2$ receptor (Simon et al., 1997). The splice variant exhibits a different pharmacology with respect to the native receptor, suggesting that there may be clear heterogeneity in the responses of tissues expressing the different proteins. Alignment of the sequences of the cytoplasmic C-terminal regions of P2X subunits shows that a motif, present in 39 of the 41 full-length P2X receptor sequences present in the data base, is conserved and that this motif is responsible for appropriate surface expression of $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_6$ receptors. In addition, this motif regulates the P2X receptors surface expression and polarization in both neurons and epithelial cells (Chaumont et al., 2004).

2.2. Electrophysiological properties

As previously anticipated, a common denominator to all P2X subtypes is a direct influx of extracellular Ca^{2+} (as well as Na^+ and/or K^+), promoted by purines through the receptor channel itself. This entry is the first event in the purinergic machinery of activation and represents a significant source of the overall intracellular Ca^{2+} pool. It then causes a membrane depolarization leading to a secondary opening of voltage-gated Ca^{2+} channels, which probably makes the most conspicuous contribution to the total intracellular Ca^{2+} influx and accumulation. This transduction mechanism does not depend on production and diffusion of second messengers within the cytosol or the membrane and, therefore, the cellular response-time is generally very rapid (within 10 ms). Homomeric $P2X_1$ receptors are cation-selective channels that show little selectivity for sodium over potassium. Extracellular calcium has little or

no inhibitory effect on $P2X_1$ receptor currents, whereas extracellular acidification inhibits currents at this receptor (Evans et al., 1996). $P2X_1$ receptors undergo fast desensitization when the agonist application is continued for more than several hundred milliseconds and recovery from desensitization is extremely slow. The desensitization is not marked at concentrations lower than, or equal to the EC_{50} concentration, but becomes prominent at concentrations above 1 μM. Mutations of positively charged residues in the extracellular loop of the human $P2X_1$ receptor can also have dramatic effects on desensitization. Homomeric $P2X_2$ receptors are permeable to calcium and the P_{Ca}/P_{Na} is ~2.5 in 5 mM external calcium; this is less than for the $P2X_1$ receptor (Evans et al., 1996) and $P2X_4$ receptor (Garcia-Guzman et al., 1997), but more than for the $P2X_3$ receptor (Virginio et al., 1998). Extracellular divalent cations have at least 2 distinct actions on the $P2X_2$ receptor. Firstly, they block the open channel and, in this case, the EC_{50} for calcium is ~5 mM, the order of effectiveness is $Mn^{2+} > Mg^{2+} > Ca^{2+} > Ba^{2+}$, and the results fit to a single binding site. Secondly, they reduce the probability of a channel being open and, in this case, the EC_{50} for calcium is about 1.3 mM, the order of effectiveness is $Ca^{2+} > Mg^{2+} > Ba^{2+} > Mn^{2+}$, and the results are best fit by the binding of 4 cations. With whole cell recording, currents at $P2X_2$ receptors decline little during agonist applications of a few seconds (Brake et al., 1994; Collo et al., 1996). For this reason, the $P2X_2$ receptor is generally described as non-desensitizing receptor. However, there is a progressive decline in the current that occurs during applications of several tens of seconds (slow desensitization). Homomeric $P2X_3$ receptors are cation-selective channels. At low concentrations (30–300 nM), ATP elicits currents that are sustained for several seconds, but with higher concentrations the currents show prominent desensitization. The desensitization occurs with a time constant of <100 ms at concentrations of 30 μM ATP (Lewis et al., 1995). Homomeric $P2X_4$ receptors are activated by ATP, but not by α,β-methylene ATP (α,β-meATP). When the ligand application is of short duration, $P2X_4$ receptors operate as cation-selective channels and the calcium permeability is relatively high. When the application of ATP is continued for several seconds, the $P2X_4$ channel becomes increasingly permeable to larger organic cations such as *N*-methyl-D-glucamine (Khakh et al., 1999; Virginio et al., 1999). The currents elicited by ATP in cells expressing the rat homomeric $P2X_5$ receptor are often several nanoamperes in amplitude and show little desensitization. The $P2X_5$ receptor is not activated by α,β-meATP, and is blocked by 8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid (suramin) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium (PPADS) at concentrations similar to those effective at $P2X_2$ receptors (Collo et al., 1996; Garcia-Guzman et al., 1996). The homomeric $P2X_6$ receptor appears to be a "silent" subunit, in the sense that no currents are evoked by ATP when it is expressed in oocytes (Le et al., 1998) or HEK293 cells (Soto et al., 1996). However, it does form heterodimers (see later). Stimulation of the homomeric $P2X_7$ receptor by ATP induces cell membrane depolarization, increase in intracellular Ca^{2+} concentration and, in most cases, permeabilization of the cell membrane to molecules up to 900 Da. The permeabilization is dependent on the intracellular Ca^{2+} and blocked by calcium chelators. In addition, mitogen activated protein kinase inhibitors (SB-203580 and PD-98059) block the permeabilization and single-channel currents (Faria et al., 2005). The currents at $P2X_7$ receptors show little or no rectification and have the following main features: require concentrations of ATP greater than 100 μM; 3′-*O*-(benzoyl-4-benzoyl)-ATP (BzATP) is 10–30 times more potent than ATP; potentiation by reduced extracellular calcium or magnesium; striking changes in time course and amplitude with repeated applications of the agonist. The ATP-evoked currents at the rat $P2X_7$ receptor is inhibited by ions such as calcium, magnesium, zinc and copper and generic P2X receptor antagonists. Nevertheless, currents are relatively insensitive to suramin (IC_{50} >300 μM at rat $P2X_7$ receptor) and PPADS (IC_{50} ~50 μM at rat $P2X_7$ receptor, IC_{50} ~3 μM at human receptor) (Surprenant et al., 1996), but the suramin analog NF279 is more potent (IC_{50} ~10 μM) (Klapperstuck et al., 2000). Calmidazolium (10 nM) blocks currents at rat $P2X_7$ receptors; 1-[*N*,*O*-*bis*(5-isoquinoline sulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) blocks currents in cells expressing the human $P2X_7$ receptor. Finally, $P2X_7$ receptor blockade by 17β-estradiol (Cario-Toumaniantz et al., 1998) and by a monoclonal antibody have also been reported (Buell et al., 1998).

2.3. P2Y receptors

P2Y receptors (Fig. 2) do not possess introns in the coding sequences, with the exception of the $P2Y_{11}$ receptor subtype, and in contrast to the P2X receptors. They are present as 308–379 amino acid proteins, with a mass of 41–53 kDa after glycosylation. They possess tertiary structures with 7 TM domains, and an extracellular N-terminus and an intracellular C-terminus. The architecture of P2Y receptors was studied by structural comparison based on sequence analysis, mutagenesis and homology modeling. The resulting phylogenetic tree delineated 2 structurally distant sub-families of P2Y receptors: the G_q-coupled subfamily, including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors, and the G_i-coupled subfamily, including $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors (see Abbracchio et al., in press). Different sets of cationic residues in the 2 subgroups appears to coordinate the binding of phosphate-bearing ligands (Costanzi et al., 2004). The 4 basic residues H121 in TM3, H266 and K269 in TM6 and R299 in TM7 near the extracellular surface might be involved in ligand binding and in coordinating the triphosphate chain of the endogenous ligand ATP. The current knowledge of the structures of P2Y receptors is derived mainly from mutagenesis studies. For instance, site-directed mutagenesis has shown that ligand recognition in the human $P2Y_1$ receptor involves individual residues of the TMs (3, 5, 6, and 7), as well as extracellular loops 2 and 3. Moreover, the binding of the negatively charged phosphate moiety of the agonists is dependent on positively charged lysine and arginine residues near the exofacial side of TM 3 and 7 (Jacobson et al., 2004).

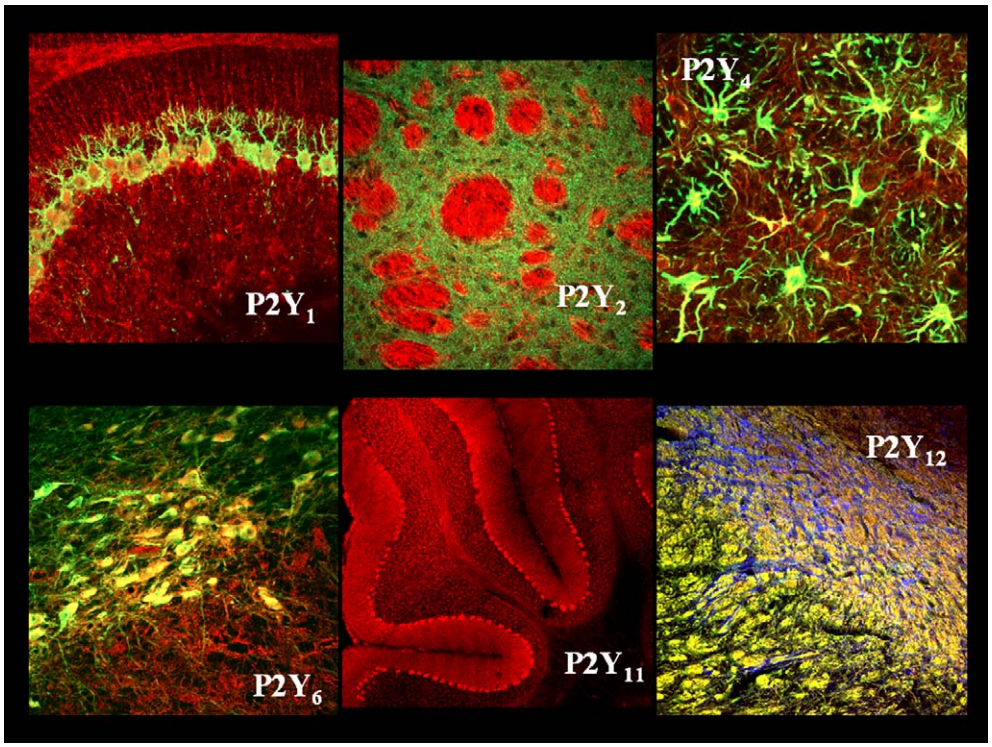

Fig. 2. Differential distribution of P2Y receptors in the CNS of adult rat. $P2Y_1$ receptor is shown on Purkinje cells in cerebellum (yellow); $P2Y_2$ receptor is observed in white matter in striatum (red); $P2Y_4$ receptor is expressed in astrocytes in globus pallidus (yellow); $P2Y_6$ receptor is present in substantia nigra pars compacta (yellow); $P2Y_{11}$ receptor is found on Purkinje cells in cerebellum (red); $P2Y_{12}$ receptor is localized on oligodendrocytes in substantia nigra pars compacta and reticulata (yellow).

2.4. Second messenger coupling

Both the P2X and the P2Y receptor subtypes modulate the level of intracellular calcium ions: whereas activation of P2X receptors results in improved permeability, mainly to extracellular Ca^{2+}, stimulation of P2Y receptor subtypes has been associated primarily in the activation of phospholipase C (PLC) leading to formation of inositol triphosphates (IP_3) (and/or modulation of adenylyl cyclase), resulting in augmentation of calcium release from intracellular stores. Nevertheless, one given P2Y receptor can couple to functionally distinct G proteins. The $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are positively coupled to PLC, via $G_{q/11}$ proteins, with the generation of IP_3 and subsequent mobilization of intracellular calcium, while the $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$ receptors are negatively coupled to adenylyl cyclase via G_i proteins. In gastric smooth muscle cells it appears that the $P2Y_2$ receptors couple to PLC-β_1 via $G\alpha_{q/11}$ and to PLC-β_3 via $G\alpha_{i3}\beta_{1\gamma 2}$-derived $\beta\gamma$ subunits. Moreover, the $P2Y_2$ receptor has also been shown to interact with α_v integrin to promote G_o-mediated chemotaxis in astrocytoma cells. The $P2Y_{11}$ receptors are dually coupled to $G_{q/11}$ and G_s proteins, activating both PLC and adenylyl cyclase. The signaling pathway of this receptor includes activation of adenylyl cyclase with a consequent increase of cAMP, which is nevertheless dependent on IP_3 generation. The $P2Y_{12}$ receptor activates phosphatidylinositol 3-kinase via $G\alpha_i$, but also RhoA and RhoA kinase. Therefore, coupling of the same P2Y receptor to distinct G proteins provides the possibility of agonist-specific signaling involving distinct active conformations of the receptors. Since P2Y receptor activation involves second messenger systems and/or G protein-mediated ionic conductance, as a consequence the cellular response time is longer (less than 100 ms) than that mediated by the P2X subtypes. Nevertheless, in recent years G protein-coupled receptors (GPCR), including P2Y receptors, have been found to modulate the activity of voltage-gated ion channels, through activated G proteins, in neurons and other excitable cells. Such actions are now well established in closing, opening or potentiating both K^+ channels and voltage-gated Ca^{2+} channels. Therefore, this becomes an important component of the signal transduction machinery of P2Y receptors, since these channel interactions can occur in very short time scales, approaching those for P2X receptors. While ion channel couplings of P2Y receptors are especially important in neurons, they have in a few cases been detected also in various other tissues (Abbracchio et al., in press). The activation of several P2Y receptors is commonly associated also with the stimulation of mitogen activated protein kinases, in particular extracellular signal regulated protein kinases 1/2. According to the cell context and the particular subtype, other classes of protein kinases, protein kinase C (PKC), calcium and PI3-K are also found to be involved to a variable extent (Communi et al., 2001).

2.5. Need for new ligands

The high molecular complexity of P2 receptors is further demonstrated by their very heterogeneous pharmacological properties. Ligand binding generally evokes conformational changes in receptors and it is possible that similar movements in the extracellular domain of P2X receptors, for instance, regulate the high-affinity binding of the adenine and triphosphate groups of ATP to these receptors. This could explain the ligand selectivity of P2X receptors, and why adenosine, polyphosphate or other nucleotides such as GTP and UTP exhibit neither agonist nor antagonist actions, because they have low affinity for the binding site and do not stabilize the conformational change (Roberts & Evans, 2004). Also for this reason, the potency of agonists can vary enormously among the different P2X receptors, with EC_{50} values ranging from 50 nM to 300 μM, depending especially on receptor subunit composition. ATP, UTP, ADP, UDP, α,β-meATP, 2-methylthioATP, β,γ-methylene ATP (β,γ-meATP), ATPγS, ADPβS, AMP, 5′-adenylyl-β,γ-imidodiphosphate, 2-cloro-ATP and BzATP are all P2 receptor agonists (Chootip et al., 2000). In contrast, the stable ATP analogs α,β-meATP and β,γ-meATP are some of the most useful agonists at P2X receptors and are generally inactive at P2Y receptors. Moreover, ATPγS is an agonist at recombinant $hP2X_2$ and $P2X_4$ receptors and it is a partial agonist at recombinant $P2X_1$ and $rP2X_2$ receptors. The most potent agonist at the endogenous $P2X_7$ receptor is BzATP, which seems to be active in $P2X_7$ receptor pore formation (Ralevic & Burnstock, 1998). Although significant advances have been made in the identification of P2X subtype selective antagonists, unequivocal pharmacological discrimination among the different subtypes is still pending and the IC_{50} values can range from subnanomolar to micromolar concentrations, subject to the different species, cell type and receptor subunit composition. A P2 receptor inhibitor, known for many years, is the trypanoside suramin (Ralevic & Burnstock, 1998; Lambrecht et al., 2002; Jacobson et al., 2004), a selective antagonist at P2 receptor versus other types of receptors, but not a universal P2 receptor antagonist. The anthraquinone-sulfonic acid derivative reactive blue 2, defined as a mixture (35:65) of the terminal ring F meta- and para-constitutional isomers, is one of the most widely used P2 receptor non-competitive antagonist, although it does not adequately discriminate between P2X and P2Y subtypes. More recently, efforts to design selective ligands based on a combination of library screening, empirical modification of known ligands, and rational design have led to the introduction of more potent and selective antagonists.

Pharmacologically, P2Y receptors can be subdivided into: adenine nucleotide-preferring receptors, mainly responding to ADP and ATP ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors) but not to UDP and UTP ($P2Y_1$ receptors are relatively insensitive to ATP but are strongly activated by ADP); uracyl nucleotide-preferring receptors, reacting to either UTP or UDP (human $P2Y_4$, $P2Y_6$ receptors); receptors of mixed selectivity ($P2Y_2$, rodent $P2Y_4$ receptors) (Ralevic & Burnstock, 1998; Communi et al., 2001); and finally into receptors responding to sugar-nucleotides UDP-glucose and UDP-galactose ($P2Y_{14}$ receptor) (Abbracchio et al., 2003). The specificity of P2Y receptor subtype-selective antagonists is still unconvincing; their IC_{50} values can range from nM to μM, depending on species and tissue localization. As with P2X receptors, the affinities for the agonists can vary from 0.1 nM (2-methythio ADP at $hP2Y_{12}$ receptor) to 200 μM (β,γ-meATP at $hP2Y_{11}$ subtype) and a variety of native agonist ligands (ATP, ADP, UTP, UDP and UDP-glucose) are still the subject of structural modification efforts to improve selectivity. Differing from P2X receptors, the absolute potency of nucleoside tri- and diphosphates for P2Y receptors is firmly dependent on the levels of receptor protein expression. Therefore, typical EC_{50} values are not easily defined for specific agonists at particular P2Y subtypes, without prior knowledge of receptor abundance.

Considering such a plethora of molecular differences among the various P2 receptor subtypes, it therefore becomes obvious that their full understanding is a very challenging topic and the design of more selective pharmacological compounds must take into account receptor abundance as well as receptor interactions. Thanks to new chemical synthesis and molecular modeling technologies, novel and potent agonists and antagonists for P2 receptors are continuously being developed (Houston et al., 2006). However, several fundamental questions still remain. From a drug discovery prospective, what is the precise structural basis of ligand specificity for a particular P2 receptor, and how can the general structures of P2 receptors be finely tuned to bind such a large and chemically diverse spectrum of ligands? From a cellular prospective, why and how are so many different receptor subtypes necessary for triggering biological properties/functions and are these receptors more than the sum of their single entities?

3. P2 receptor localization

The biological complexity and heterogeneity of P2 receptors (Figs. 1 and 2) is not uniquely of a molecular and pharmacological nature, as described above, but is further accomplished at a cellular and subcellular level.

3.1. Multiple receptors on single cells, including stem cells

A first indication of such sophistication is that multiple P2 receptor subtypes are concomitantly present on most mammalian cells, mediating both short-term and long-term biological effects. For instance, both extracellular ATP-gated P2X channels and P2Y receptors are widely expressed in many cell types (see Burnstock & Knight, 2004). For example, from a combination of Northern blotting, RT-PCR and in situ hybridisation for mRNA, immunohistochemistry and Western blotting for protein, and calcium imaging and pharmacological profiles: astrocytes express $P2X_{1-7}$ and $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$; vascular endothelial cells, $P2X_1$, $P2X_4$, $P2Y_1$ and $P2Y_2$; retinal ganglion cells $P2X_{1-5}$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$; alveolar type II cells $P2X_4$ and $P2Y_2$; tracheal epithelial and goblet cells $P2X_4$, $P2X_7$, $P2Y_1$ and $P2Y_2$; nasal epithelium $P2X_2$, $P2Y_2$ and $P2Y_6$; interstitial cells of Cajal $P2X_2$ and $P2X_5$; colon smooth muscle $P2Y_1$, $P2Y_2$ and $P2Y_6$; hepatocytes $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{13}$; cholangiocytes

$P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$; mesangial cells $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$; epithelial cells in proximal tubules $P2X_4$, $P2X_5$, $P2Y_1$, $P2Y_2$ and $P2Y_6$; epithelial cells in distal convoluted tubules $P2X_{1-5}$ and $P2Y_4$; smooth muscle of urinary bladder $P2X_1$, $P2X_2$, $P2X_4$ and $P2Y_1$; Sertoli cells in testes $P2X_4$, $P2X_7$, $P2Y_1$ and $P2Y_2$; placental trophoblasts $P2X_1$, $P2X_2$, $P2X_4$, $P2X_7$, $P2Y_2$ and $P2Y_6$; thymocytes $P2X_1$, $P2X_2$, $P2X_6$, $P2X_7$, $P2Y_1$ and $P2Y_2$; alveolar macrophages $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{12}$; eosinophils $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$; dendritic cells $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$; ventricular myocytes $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, $P2Y_1$ and $P2Y_2$; aortic smooth muscle $P2X_1$, $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$; mesenteric artery smooth muscle $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$; platelets $P2X_1$, $P2Y_1$ and $P2Y_{12}$; pancreatic acini cells $P2X_1$, $P2X_4$, $P2Y_2$ and $P2Y_4$; osteoclasts $P2X_2$, $P2X_4$, $P2X_7$, $P2Y_1$ and $P2Y_2$; brown adipocytes $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$; sympathetic (superior cervical ganglia) neurons $P2X_{1-7}$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$, and sensory (dorsal root ganglia, DRG) neurons $P2X_{1-6}$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors.

In many tissues there is evidence that shows multiple receptors on single cells, in other tissues it is possible that subpopulations of cells express different receptors. In some cells, apical and baso-lateral membranes express different receptor subtypes, for example lung and kidney epithelial cells.

The mRNA for several P2 receptors ($P2Y_1$, $P2Y_2$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2X_1$ and $P2X_4$ receptors) was found to be expressed in CD34+ stem and progenitor cells, therefore showing a role in differentiation and proliferation, and implicating a potential role of P2 receptors in hematopoietic lineage and progenitor/stem cell function (Wang et al., 2004). In addition, stimulation of CD34+ cells with extracellular nucleotides causes a fast release of Ca^{2+} from intracellular stores and an increase in ion fluxes across the plasma membrane. Functionally, ATP and, to a greater extent, UTP act as potent early acting growth factors for hematopoietic stem cells, in vitro, strongly enhancing the stimulatory activity of several cytokines on clonogenic CD34+ and lineage-negative CD34−. Extracellular nucleotides may therefore provide a novel and powerful tool to modulate hematopoietic stem cells functions (Lemoli et al., 2004). A P2Y-like receptor for UDP-conjugated sugars ($P2Y_{14}$ receptor) has been identified on primitive quiescent cells in the hematopoietic lineage restricted to bone marrow. It mediates primitive cell responses to specific hematopoietic microenvironments and extends the already known immune system functions of P2Y receptors to the stem cell level. These data therefore suggest a new class of receptors, the P2 receptors, participating in the regulation of the stem cell compartment (Lee et al., 2003). Although neural progenitor cells (NPC) may provide a source of new neurons to alleviate neural trauma, little is known about their expression of functional P2 receptors. ATP and BzATP evoke an inward current and membrane depolarization in single-cell preparations of both undifferentiated and differentiated adult hippocampal NPC, from 0 to 12 days in vitro. In Fura-2-loaded undifferentiated NPC, ATP and BzATP evoke a transient increase in the intracellular free Ca^{2+} concentration, which is dependent on extracellular Ca^{2+} and is inhibited reversibly by PPADS (Hogg et al., 2004).

3.2. Subcellular location including electronmicroscopy

The ubiquitous cellular expression of P2 receptor subtypes further raises the possibility that P2 receptors are distributed between plasma membrane and intracellular cytosolic compartments, dynamically moving in and out from these structures, often depending upon functional, developmental state, presence of agonists or antagonists in the extracellular space. Various P2 receptors are indeed found not only in the plasmalemma, but also in the cytoplasm, endoplasmic reticulum, Golgi complex, nuclear envelope, endosomes and neurosecretory granules (Loesch & Burnstock, 2001; Atkinson et al., 2002). The existence of $P2Y_1$-like and $P2Y_2$-like receptors in mitochondria of hepatocyte was reported, where they might regulate Ca^{2+} uptake through the uniporter. Mitochondrial $P2Y_1$ receptors stimulate Ca^{2+} uptake, whereas $P2Y_2$ receptor activation inhibit Ca^{2+} uptake (Belous et al., 2004). Furthermore, P2 receptors are present on synapses, axons and dendrites, as well as on the nuclear membrane of neurons and glial cells. For instance, the expression of $P2X_2$, $P2X_{2\text{-}2}$ and $P2X_5$ receptor subunits was demonstrated in rat thoracolumbar neurons. Laser scanning microscopy has revealed that whereas $P2X_2$ receptor immunoreactivity is largely distributed over the neuronal body, $P2X_5$ receptor immunoreactivity is most distinctly located close to the nucleus (Schadlich et al., 2001). Furthermore, several P2 receptor subtypes can associate to specialized membrane compartments such as synaptic vesicles, “lipid rafts” and “caveolae” (Deuchars et al., 2001; Vacca et al., 2004). For instance, the presence of $P2X_3$ receptors in lipid rafts is well established for protein sorting in the trans-Golgi network, in polarized epithelial cells and neurons. Axonal protein sorting, correct neuronal polarization, clustering in somatic spines, synapse formation, critically depend upon lipid rafts integrity (Ledesma et al., 1998; Brusès et al., 2001). In this respect, it is appealing to speculate that the presence of P2 receptors in lipid rafts might contribute to the correct localization of these neurotransmitter receptors (Hervàs et al., 2003; Vacca et al., 2004). There is also evidence that association of TM receptors and signaling molecules with lipid rafts provides an enriched environment for protein–protein interactions necessary for signal transduction. It is well documented that receptor tyrosine kinases co-localize in rafts with adapters and downstream kinases needed for their signaling. Moreover, several cytoplasmic protein kinases, including different PKC isoforms, are located in rafts (Lisanti et al., 1994; Oka et al., 1997) and the enrichment of these kinases within rafts provides a mechanism for the modulation of neurotransmitter receptor function. Interestingly, there is a PKC consensus sequence in the amino-terminal tail of all P2X receptor family members and it was also reported that PKC phosphorylation modulates the desensitization kinetics of the $P2X_2$ receptor channel (Boué-

Grabot et al., 2000). Several membrane receptors translocate into lipid rafts after ligand binding, while others move in the opposite direction. The functions of such a dynamic receptor localization have been generally associated with signaling modulation and receptor desensitization or internalization (Zajchowski & Robbins, 2002). In cerebellar granule neurons cultures, however, the $P2X_3$ receptor appears to be constitutively localized in lipid rafts and any re-localization after stimulation with the natural ligand ATP or with the more selective $P2X_3$ receptor ligand α,β-meATP has so far been unsuccessful (Vacca et al., 2004).

The occurrence of P2 receptors on precise subcellular compartments has encouraged higher resolution studies using electronmicroscopy. For instance, $P2X_1$ and $P2X_2$ receptors appear in both postsynaptic and presynaptic sites (Vulchanova et al., 1996; Llewellyn-Smith & Burnstock, 1998; Loesch & Burnstock, 1998; Loesch et al., 1999), but several P2X subunits are only present at postsynaptic specialization in cerebellum and of Schaffer collateral synapses in hippocampus. Analysis of their tangential distribution at the synapse showed that these receptors are localized at peripheral portions of the postsynaptic specialization (Rubio & Soto, 2001). The distribution of the $P2X_2$ and $P2X_6$ subtypes was more extensively analyzed at the electronmicroscopy level in the rat hypothalamo-neurohypophysial system. The $P2X_2$ receptor is localized in subpopulations of neurons, neurosecretory and non-neurosecretory axons and dendrites in paraventricular and supraoptic nuclei, and in neurohypophysis pituicytes and subpopulation of neurosecretory axons (Loesch et al., 1999). The $P2X_6$ receptor was instead visualized in sub-populations of neurosecretory cell bodies, neurosecretory and non-neurosecretory axons and dendrites of neurons in the paraventricular and supraoptic nuclei and in pituicytes and neurosecretory and non-neurosecretory axons of the neurohypophysis. Moreover, using the immunogold-silver method, electron-dense particles labeling $P2X_6$ receptors were found in the cytoplasm, endoplasmic reticulum, Golgi complex and neurosecretory granules (Loesch & Burnstock, 2001). At the electron microscope level, $P2X_3$ receptor-immunoreactivity in somata was concentrated in and around membranous organelles, including the endoplasmic reticulum and Golgi apparatus. In axon terminals, $P2X_3$ receptor-immunoreactivity was observed in small round vesicles (Llewellyn-Smith & Burnstock, 1998). Ultrastructural studies of the $P2X_4$ receptor distribution in the dorsal vagal complex of the brainstem in adult rats, revealed that $P2X_4$ receptor immunoreactivity is present in neurons at both pre- and post-synaptic sites as well as in glial cell processes and somata. $P2X_4$ receptor is adjacent to plasma membranes, as well as located internally in membrane bound structures resembling endosomes (Ashour & Deuchars, 2004). $P2X_5$ receptor immunoreactivity was most distinctly located close to the nucleus. Immunoelectron microscopy also revealed the $P2X_7$ receptor localization on synapses, dendrites, as well as on the nuclear membrane of neurons and glial cells (Franke et al., 2004b). An ion channel spanning the nuclear envelope between the cytoplasm and the nucleus was proposed to be regulated by an ATP-binding receptor of the $P2X_7$ receptor subtype. Activation of this nuclear $P2X_7$ receptor by ATP in the cytoplasm may therefore be a mechanism by which cellular energy can be coupled to changes in gene expression (Atkinson et al., 2002).

3.3. Plasticity of receptor expression, including development

The concomitant expression of multiple P2 receptor subtypes on most mammalian cells cannot be considered a static event, rather a dynamic process always reaching a new equilibrium as a function of the unpredictably mutating extracellular environment. For example, there are two phenotypes of smooth muscle, the contractile phenotype and the synthetic (proliferative) phenotype. $P2X_1$ receptors are strongly expressed by the contractile phenotype, but in the synthetic phenotype expressed in culture, the mitogenic $P2Y_1$ and $P2Y_2$ receptor transcripts were upregulated 342- and 8-fold, respectively, whereas the contractile $P2X_1$ receptors were totally downregulated; $P2Y_4$ and $P2Y_6$ receptors were unchanged (Erlinge et al., 1998). Upregulation of $P2X_1$ and $P2Y_2$ receptor mRNA in heart of rats with congestive heart failure has been reported (Hou et al., 1999). In the vasculature, the expression of $P2X_1$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors was studied during inflammation. The expression of $P2X_7$ and $P2Y_1$ receptors on macrophages and giant cells increases during the course of the entire inflammatory reaction, while the expression of the $P2Y_2$ receptor on macrophages is maximal at day 5. The expression of $P2X_1$ receptor remains instead at a constant low level. The upregulation of $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors over time therefore suggests a regulatory function for these receptors in inflammation (Luttikhuizen et al., 2004). Microglial cell lines express multiple P2 receptors at the mRNA and/or protein level and most of them (at least the $P2X_7$ and $P2Y_1$, $P2Y_{2/4}$, $P2Y_6$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptor subtypes) are coupled to $[Ca^{2+}]_i$ increases. Nevertheless, microglia activation in mouse and rat brain upregulates the $P2Y_6$ and $P2Y_{14}$ receptors, downregulates the $P2X_7$ receptor, without affecting the $P2Y_1$, $P2Y_2$ or $P2Y_4$ receptors (Bianco et al., 2005). It is possible that modulation of microglial cell function via subtype-selective P2 receptor ligands may thus open up new strategies in the therapeutic management of inflammatory neurological diseases characterized by abnormal microglia response. Using Northern blot analysis it was also demonstrated that the $P2Y_{12}$ receptor is expressed predominantly in small cells distributed throughout the brain, including the hippocampus, and its distribution is modulated in microglia during development and after facial nerve axotomy (Sasaki et al., 2003). In both cases, the increase in the number of $P2Y_{12}$ receptor-positive cells would suggest that chemotaxis of microglia to axotomized motor neurons could promote regeneration through a potential role of this receptor (Sasaki et al., 2003). Extracellular nucleotides can regulate proliferation, differentiation and apoptosis of cancer cells, through different P2 receptor subtypes (White & Burnstock, 2006). For instance, expression and activation of the metabotropic $P2Y_2$ receptor can lead to an increase in cell number in most

cancer types, whereas activation of the $P2Y_1$ receptor and ionotropic $P2X_5$ and $P2X_7$ receptors leads to a decrease in cell number. Moreover, it is known that higher expression of the metabotropic $P2Y_4$ receptor causes commitment to differentiation and cell death of human neuroblastoma cells (Cavaliere et al., 2005). Thus, it has been clearly demonstrated that different receptor subtypes can be present on the same cell in different amounts, and that the varying quantity of receptors can have opposite effects on cell number. This arrangement suggests that the control of cell proliferation by extracellular nucleotides may be regulated by a critical balance of the activities of the different receptor subtypes mediating mitogenic or apoptotic processes.

Purines and pyrimidines are involved in signalling in very primitive systems and it is, perhaps, not surprising that so many different receptor subtypes have been explored during evolution. ATP plays key roles from the very beginnings of life, that is, the moment of conception. ATP is indeed obligatory for sperm movement, is a trigger for capacitation (the acrosome reaction necessary to fertilize the egg) and promotes a rapid increase in ion permeability of the fertilized egg membrane. ATP-activated spermatozoa show very high success rates in fertilization tests, this strongly suggests that ATP is a key sperm-to-egg signal in the process of fertilization (Burnstock, 1996). Moreover, expression of various P2 receptor subtypes was studied in the apical, lateral and basal plasma membranes of rat uterine epithelial cells during early pregnancy to the time of implantation on day 6. Labeling for each P2X subtype was seen in the apical, lateral and basal surfaces, except for the $P2X_2$ receptor, which was only observed in the basement membrane. At the time of implantation on day 6, this pattern was dramatically altered, with apical expression markedly increased for most subtypes, while the lateral and basal signals were markedly reduced; $P2X_2$ receptors were strongly expressed on the basal and apical surfaces, while $P2X_4$ receptors became repressed in all areas. Therefore, the spatial and temporal changing expression of the P2X receptors is a significant factor in the regulation of events during early pregnancy (Slater et al., 2000). Moreover, the study of knockout mice has demonstrated that P2X receptors play important roles in the neurogenic control of smooth muscle contraction, in pain and visceral perception, and in macrophage functions (Boeynaems et al., 2005). All P2X receptor subtypes, except for the $P2X_1$ receptor, were strongly represented in the developing rat brain. In vitro data also indicate that ATP via its P2X and P2Y receptors can shape hippocampal connectivity during development (Heine et al., 2006). Moreover, ATP was shown to inhibit motor axon outgrowth during early embryonic neurogenesis, most likely via the $P2X_3$ receptor. It was speculated that $P2X_7$ receptors may be involved in programmed cell death during embryogenesis and that $P2X_4$, $P2X_5$ and $P2X_6$ receptors might be involved in postnatal neurogenesis (Cheung et al., 2005). Changes in $P2Y_2$ receptor localization on adrenaline- and noradrenaline (NA)-containing chromaffin cells in the rat adrenal gland during development and aging were also demonstrated (Afework & Burnstock, 2005).

4. Subunit association and receptor cross talk

The biological complexity of P2 receptors is further augmented, if we consider that both P2X and P2Y subtypes can form homomers and heteromers (Torres et al., 1999; Nakata et al., 2004), and that the composition of the oligomers profoundly affects the biological response of these receptors. Different subtype combinations thus yields different receptor characteristics, allowing increasing diversities in agonist and antagonist selectivity, transmission signaling, channel and desensitization properties: this certainly helps to explain why and how so many different receptor subtypes are indeed combined on a given cell for triggering such a vast plethora of purinergic functions.

4.1. P2X receptor heteromultimers

Quaternary structural features of P2X receptors have been predicted from the amino acid sequences of cloned P2X proteins. Since P2X subunits possess 2 TM domains such as the inward rectifying potassium channels, a tetrameric organization was originally anticipated. However, biochemical analyses of recombinant $P2X_1$ and $P2X_3$ receptors revealed an unexpected trimeric subunit organization (Nicke et al., 1998) that is consistent with functional studies (Jiang et al., 2003). Further biochemical evidence indicates that the receptors occur as stable trimers or hexamers of 3 or 6 subunits (Nicke et al., 1998). Hetero-multimeric assemblies of $P2X_{2/3}$ receptors (Radford et al., 1997), $P2X_{4/6}$ receptors (Le et al., 1998), $P2X_{1/5}$ receptors (Torres et al., 1998), $P2X_{2/6}$ receptors (King et al., 2000), $P2X_{1/2}$ receptors (Brown et al., 2002), and most recently $P2X_{1/4}$ receptors (Nicke et al., 2005) have been described, while $P2X_7$ receptor does not apparently co-assemble with any other subunit (Torres et al., 1999). Intracellular rat and human $P2X_2$ subunits exhibit distinct assembly states; $rP2X_2$ receptors exist as individual homotrimers and clusters of homotrimers at the plasma membrane; $hP2X_2$ receptors, subjected to virtually identical conditions (treatment with DTT and exposure to urea), were instead demonstrated to exist as dimers and monomers but also in a trimeric state formed by both dimers and monomers (Aschrafi et al., 2004). Finally, the $P2X_2$ subunit was incorporated not only into homomeric, but also in heteromeric assemblies with the $P2X_1$ subunit ($P2X_{1+2}$ receptor) (Brown et al., 2002) or the $P2X_3$ subunit ($P2X_{2+3}$ receptor) (Lewis et al., 1995).

$P2X_1$ and $P2X_2$ receptor subunits have been coexpressed in defolliculated Xenopus oocytes and the resultant receptors studied under voltage clamp conditions. Coexpression yielded a mixed population of homomeric and heteromeric receptors with a subpopulation of novel pH-sensitive P2X receptors showing identifiably unique properties that indicate the formation of heteromeric $P2X_{1/2}$ ion channels. It has been claimed that trimeric $P2X_{1/2}$ receptors incorporates 1 $P2X_1$ and 2 $P2X_2$ subunits.

Based on disulfide bond formation between engineered cysteine residues, it has recently been suggested that a trimeric $P2X_{2+3}$ receptor would have the composition $P2X_2(P2X_3)_2$

(Jiang et al., 2003). In certain sensory neurons, sympathetic ganglion cells, and brain neurons, a coexpression of heteromeric $P2X_{2/3}$ receptor often occurs, as shown by coimmunoprecipitation experiments. The relatively slow desensitization of these currents is one of the defining features of the expressed $P2X_{2/3}$ receptor heteromers. The $P2X_{2/3}$ heteromer shares with the homomeric $P2X_3$ receptor the high sensitivity to block by TNP-ATP, as well as PPADS and suramin. Ip_5I is much more potent to block $P2X_1$ and $P2X_3$ homomers than to block the $P2X_{2/3}$ heteromers and is therefore useful to distinguish between $P2X_3$ and $P2X_{2/3}$ receptors.

Co-injection of $P2X_1$ and $P2X_4$ subunits into Xenopus oocytes showed that both subunits were present in trimeric complexes of the same size. Voltage clamp experiments revealed functional P2X receptors with kinetic properties resembling homomeric $P2X_4$ receptors and a pharmacological profile similar to homomeric $P2X_1$ receptors. Preliminary results show that the $P2X_1$ receptor from the vas deferens and the $P2X_4$ receptor from salivary gland form complexes of the same size as the recombinant trimeric complexes expressed in oocytes.

$P2X_1$ and $P2X_5$ subunits can be coimmunoprecipitated and the defining phenotype of this heteromer is a sustained current evoked by α,β-meATP, which is not seen for either of the homomers when expressed separately. Cells expressing the heteromeric $P2X_{1/5}$ receptor provide responses to ATP that have several unique features (Haines et al., 1999). Firstly, they are more sensitive to ATP than those with homomeric receptors, and concentrations as low as 3 or 10 nM evoke measurable currents. Secondly, the kinetics of the response are distinct: at the very low concentrations, the currents are sustained over several seconds with little desensitization, but when the concentration exceeds 300 nM, they show an initial peak that rapidly declines and is followed by a sustained component. Thirdly, repeated applications of agonist at intervals of 10 s give quite reproducible inward currents. Lastly, currents are inhibited by either an increase or a decrease of the extracellular pH (Surprenant et al., 2000). They are little affected by increasing the extracellular calcium concentration to 30 mM; this is similar to the $P2X_1$ homomer and different from the $P2X_5$ homomer. The sensitivity to suramin and PPADS is similar to that of each of the constituent homomers, but low concentrations of PPADS (100 nM) also potentiate the "plateau" phase of the current. However, the sensitivity to TNP-ATP is intermediate between the sensitive homomeric $P2X_1$ receptor and the insensitive homomeric $P2X_5$ receptor.

Heteromeric $P2X_{2/6}$ receptors have been found to coimmunoprecipitate after expression in HEK293 cells (Torres et al., 1999). At pH 6.5 the inhibition of the current by suramin is clearly biphasic; one component has the high sensitivity of homomeric $P2X_2$ receptors ($IC_{50} \sim 80$ nM) (King et al., 2000), whereas the other component is less sensitive ($IC_{50} \sim 2$ μM) (King et al., 1997). $P2X_{2/6}$ receptors are prominently expressed by respiratory neurons in the brain stem.

Heteromeric $P2X_{4/6}$ receptors form a channel when coexpressed in oocytes (Khakh et al., 1999). The principal functional evidence for coexpression is that currents elicited by ATP are larger in oocytes after injection of mRNAs for both $P2X_4$ and $P2X_6$ receptors than after injection of $P2X_4$ receptor alone (Le et al., 1998). However, the phenotype of the heteromer differs only in minor respects from that of $P2X_4$ receptor homomers.

4.2. P2Y receptor oligomeric interactions

It is well known that GPCR can directly associate as either homo- or hetero-oligomers, producing a different function from that evoked by the original receptors. For instance, dopamine D1 and adenosine A_1 receptors form functionally interacting heteromeric complexes (Gines et al., 2000). The A_{2A} receptor exists in living cells as an oligomer, either homomeric or heteromeric with D2 receptor, providing a molecular basis for adenosine–dopamine antagonism at the membrane or cellular level (Canals et al., 2003; Kamiya et al., 2003). Moreover, accumulating evidence suggests that activation of one particular signaling pathway of a GPCR can either amplify or inhibit the intracellular pathway of another. For example, a recent study reported that stimulation of the PLC-coupled $P2Y_2$ receptor specifically inhibited the β2-adrenergic receptor-mediated cAMP production via G_i proteins, and a close colocalization of the $P2Y_2$ receptor and β2-adrenergic receptors was proposed to explain the rapid and selective interaction between these two receptors (Suh et al., 2001). This confirms that two different receptors could be coupled to each other via direct intramembrane association or indirect linkage through an anchoring protein, resulting in cross-regulation between receptor-mediated signaling. G protein-coupled purinergic receptors are also found to oligomerize and to alter their own pharmacology. Adenosine receptors of the A_1 subtype are able to act synergistically with P2Y receptors (Fredholm et al., 2003) and to form a heteromeric complex with $P2Y_1$ receptors (Yoshioka et al., 2002). Heteromerization between A_1 and $P2Y_1$ receptors thus generates an adenosine receptor with a P2Y-like receptor agonistic pharmacology. In fact, adenosine 5′-*O*-(2-thiodiphosphate), a potent $P2Y_1$ receptor agonist, binds the A_1 pocket of the $A_1/P2Y_1$ receptor complex and inhibits adenylyl cyclase activity via $G_{i/o}$ protein. This hetero-oligomerization between adenosine receptors and $P2Y_1$ receptors might therefore be one of the mechanisms for the adenine nucleotide-mediated inhibition of neurotransmitter release (Nakata et al., 2005). Moreover, a constitutive, physical interaction between the $P2Y_2$ receptor and $G_{\alpha 16}$ in the hematopoietic K562 cells confirms the postulated link between the hematopoietic cell-specific G protein $G_{\alpha 16}$ and the $P2Y_2$ receptor, implying that G protein-dependent signal transduction may not only be regulated by agonist binding to the receptor, but may also be controlled by the extent of pre-existing receptor-G protein complexes (Kotevic et al., 2005). These mechanisms generate further functional and structural heterogeneity among P2 receptors, providing evidence to help explain how different cells are able to evoke diverse responses in different tissues, upon the same kind of stimulus (Tanoue et al., 2004). Homomeric and heteromeric complexes occurring directly between various P2Y subunits were also recently reported (D'Ambrosi et al., 2005). Both rat and human endogenous $P2Y_4$ receptors, originating either from cell lines

or primary neurons from both peripheral and CNS, can in fact display molecular masses corresponding to monomeric, dimeric and oligomeric structures. This occurs also for the heterologous $P2Y_4$ receptor, transiently transfected in the neuroblastoma SH-SY5Y cell line (D'Ambrosi et al., 2005). Homodimers of the human $P2Y_1$ receptor have recently been shown by fluorescence resonance energy transfer (FRET) confocal microscopy, and dimerization to increase with exposure to agonist (Choi et al., 2005). These results confirm what is already established for other GPCR, that they can directly associate as either homo- or hetero-oligomers. In particular, as documented above, adenosine A_1 and A_{2A} receptors form homodimers, and these same subunits heterodimerize with dopamine D1 and D2 receptors or with metabotropic glutamate receptors. In conclusion, since the composition of the oligomers profoundly affects the biological response of P2 receptors, we believe that the quaternary structure of P2Y receptors might, in part, help to explain how these metabotropic subtypes can trigger so many different functions.

4.3. Receptor cross talk

In the last few years, increasing evidence of functional cross talk between different types of membrane receptors has been reported, based on co-localization within the same membrane domain that would facilitate such functional interactions. Recent studies indeed displayed interplay particularly of P2 proteins with other classes of receptors. Mammalian transmitter-gated ion channels consist of 3 families: Cys-loop channels for acetylcholine, γ-amino butyric acid (GABA), glycine and serotonin; glutamate-gated channels (kainate, AMPA, and NMDA); and the ATP-gated P2X channels. There is little sequence, topological or structural similarity between members of the 3 families, leading to the assumption that they should act independently in neurons and synapses. Nevertheless, molecular and/or functional interactions have been demonstrated between P2X receptors and both these two other families of transmitter-gated ion channels. Sokolova et al. proposed a negative cross talk between GABA and P2X receptors in rat DRG neurons (Sokolova et al., 2001). The $P2X_3$ receptor, in particular, is functionally modulated by $GABA_B$ receptor activation, both in DRG neurons (Sokolova et al., 2001) and in midbrain synaptosomes (Gomez-Villafuertes et al., 2003). Moreover, it was recently reported that $P2X_3$ receptor stimulation regulates ephrinB ligand re-distribution at the plasma membrane of DRG neurons (Battaglia et al., 2003). Interestingly, both $GABA_B$ receptor (Becher et al., 2001) and ephrinB ligands (Bruckner et al., 1999) have been identified as raft-resident proteins, with $P2X_3$ receptors (Vacca et al., 2004). $P2X_3$ receptor localization in lipid rafts could therefore be an opportunity for this receptor to preferentially interact with regulatory proteins and to be functionally coupled to additional receptors within the same microenvironment. Nicotinic and P2X receptor channels are also known to functionally cooperate and coactivate, resulting in cross-inhibition of one or both channel types. In particular, $P2X_2$ receptor and α4β2 nicotinic channels form a molecular partnership with close spatial apposition in the plasma membrane of living cells, influencing each other when coactivated (Khakh et al., 2005). In smooth muscle cells of the vascular and genitourinary systems, the vasoactive mediator 5-hydroxytryptamine (5-HT), via the 5-HT2A metabotropic receptor, regulates the desensitization kinetics of $P2X_1$ receptor responses, by increasing the rate of recovery. Reconstituting the potentiation of $P2X_1$ ATP-gated currents by 5-HT2A receptors in the Xenopus oocyte expression system, it was also shown that this modulation depends on the activation of novel PKC isoforms and protein kinase D (also named PKCμ) downstream of PLCβ (Ase et al., 2005).

Rubio and Soto showed by immunogold labeling that synapses expressing P2X receptors are also glutamatergic in the cerebellum and hippocampus (Rubio & Soto, 2001). Moreover, it was proposed that glutamate and nucleotide receptors might be considered as separate components of a single functional receptor unit acting in synergism to regulate the efficiency of the synaptic transmission (Volonté & Merlo, 1996; Amadio et al., 2002, 2005). Functional relationship and co-localization at the membrane level between ionotropic $NMDAR_1$ receptors and metabotropic $P2Y_4$ receptors was also shown in cerebellar granule neurons and human neuroblastoma cells, where these receptors are oppositely modulated during glucose starvation (Cavaliere et al., 2004b). Stimulation of $G_{\alpha q}$-coupled metabotropic glutamate receptor 1α, $P2Y_1$ or $P2Y_2$ receptors co-expressed with $P2X_1$ receptors in Xenopus oocytes was demonstrated to evoke calcium-activated chloride currents and potentiate subsequent $P2X_1$ receptor-mediated currents, suggesting that in vivo fine-tuning of $P2X_1$ receptors by GPCR may contribute to cardiovascular control and haemostasis (Vial et al., 2004b).

An additional class of receptors found to be functionally coupled to P2X proteins in sensory fibers and neurons of rat are opioid receptors. Both fast (mediated by $P2X_3$ receptors) and slow (mediated by $P2X_{2/3}$ heteromeric receptors) responses to ATP were inhibited by opioids in a characteristic biphasic manner: an initial short phase of potentiation (lasting for 300–400 s) followed by a long-lasting inhibition of the response (Chizhmakov et al., 2005). A functional cross talk mediated by ATP, potentially through P2 receptors, is exemplified by progesterone and estrogen receptors. Activation and molecular transformation (from a 4-S receptor form into a 5-S form when analyzed on sucrose gradient) of progesterone receptor complex by ATP was demonstrated, although the steroid-binding properties remained intact and the ATP-activated progesterone-receptor complex lost the ability to aggregate. The rat uterine estradiol-receptor complex was equally activated by ATP (Moudgil et al., 1981). Moreover, mechanisms of postjunctional synergism between ATP and NA were also shown in isolated guinea pig vas deferens. Whereas prior exposure to ATP has no significant effect on NA-mediated contractions, NA concentration-dependently enhances ATP-induced contractions (Smith & Burnstock, 2004). A parallel synergistic action of ATP and NA was also shown in the hypothalamus to cause vasopressin release (Song & Sladek, 2006). Functional cross talk between A_{2A} and P2 receptors in modulating pituitary

hormone synthesis and secretion (Zhao et al., 2006), or between various adenosine receptors and $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor subtypes in controlling the $[Ca^{2+}]_i$ response in cultured astrocytes (Alloisio et al., 2004) were also demonstrated. Moreover, purinergic signalling and particularly the interplay between P2Y and P1 receptors is known to mediate the inhibition of osmotic swelling of retinal glial cells, although also involving neuropeptide Y and group II mGlu receptors, as well as extracellular ATP degrading enzymes (Uckermann et al., 2006). ATP can be enzymatically degraded to adenosine, the activity of P1 and P2 purinergic systems may be highly interdependent. The ecto-nucleotidase cascade would therefore assume a key role in balancing the actions of these two neuromodulatory systems, not only by regulating extracellular nucleotide and nucleoside levels (Zimmermann, 2000), but also by representing an additional class of receptor interacting proteins. Adenine has been recently identified as the endogenous ligand of an orphan rat GPCR with the highest expression in the small neurons of the DRG, therefore suggesting a role in nociception (Bender et al., 2002), and potent neurotrophic effects of adenine have been described in primary cultures of rat cerebellar Purkinje cells (Yoshimi et al., 2003). Thus, like P1 and P2 receptors, adenine receptors also highly expressed in rat brain (Gorzalka et al., 2005) may likely be targets for multiple receptor interactions.

A cross talk between ionotropic $P2X_7$ receptors or metabotropic $P2Y_4$ receptors and the p75 low affinity NGF receptor and between $P2Y_2$ receptors and TrkA (Arthur et al., 2005) has been demonstrated. In addition, the interaction between extracellular ATP and nerve growth factor in mediating either neuronal differentiation in PC12 cells (D'Ambrosi et al., 2000, 2001) or hypoglycemia-induced cell death in cerebellar granule neurons (Cavaliere et al., 2002) has been described. This molecular interplay of P2 receptor proteins with neurotrophic factor receptors is conceptually noteworthy and it might have intriguing implications for the response of cells to trauma, ischemia, tumor and other acute or chronic neurodegenerative insult.

5. P2 receptor web and fine-tuning

Some P2 receptor subtypes are very similar, others have quite different properties. For instance, $P2Y_{12}$ and $P2Y_{13}$ receptors have the same preference for endogenous agonists (particularly ADP) and couple to the same intracellular signal transduction pathway, hence implicating on a first analysis that cellular responses might be analogous, whether a cell expresses one or the other receptor subtype (or a mixture of both). Moreover, $P2Y_1$ receptors are coupled to stimulation of nitric oxide synthesis (Kittner et al., 2003), for instance in endothelial cells, but also to inhibition of ion fluxes in glutamatergic cortical neurons (Guzman et al., 2005) or to activation of excitatory conductance in hippocampal interneurons (Kawamura et al., 2004). Nevertheless, the ADP sensitive $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors couple to different signal transduction pathways (G_q- vs. G_i-coupled), hence demonstrating differences in down stream events. As a result, expression of similar or distinct membrane bound receptors and/or triggering of similar or distinct intracellular signal transduction pathways must not be heralded as the unique criteria to explain what contributes to determine each specific cellular response to extracellular nucleotides. Nature is likely to be much more complex and from such a vast biological scenario and molecular complexity and diversity among P2 receptor subunits, it clearly emerges that: (i) any classification of P2 receptors must be associated to a dynamic process; (ii) multiple P2 subtypes are required for gaining biological functions; (iii) P2 proteins are more than the sum of their single entities.

As a consequence, we would like to propose a new way of looking at P2 receptors that takes into account all these features and, moreover, to introduce the use of a simple mathematical equation to explain how the molecular variety of P2 receptors can be combined on a cellular membrane to generate and possibly optimize all the specific functions that involve these receptors. Thus, P2 receptors should more properly be regarded as a "combinatorial receptor web". This will allow not only to better dissect their heterogeneity but, at the same time, to integrate and upgrade their assortment. We emphasize that the concept of receptor web is not a novel classification for P2 receptor proteins, but is, instead, a dynamic architecture of these same receptors demonstrating high economic efficiency. It consists in the energy-saving principle of simultaneously recruiting different combinations of fundamental building blocks, the P2 proteins, to attend to extra functions on a single cell: receptor "a" or its multiple aggregation states "aa", or "aaa" and so on, for function "A", receptor "b", or its multiple aggregation states, for function "B", receptors "a+b", or their multiple aggregation states, for function "C", and so on. Let's assume to have a number "n" of distinct P2 receptor subtypes (in particular: $n=7$ for ionotropic P2X receptors; $n=8$ for metabotropic P2Y receptors), and that the simultaneous presence of a combination (set) of different receptor subtypes on a given cell membrane will generate a biological task (function) "f", in a way that different sets of subtypes will generate different functions. We can easily enumerate all the possible different purinergic functions originating from this system. If we consider "m" distinct P2 receptor subtypes, with $1 \le m \le n$, the number of combinations in which we can choose the m receptors from the total number "n" of distinct P2 receptor subtypes, is given by N_m.

$$N_m = \frac{n!}{m!(n-m)!}$$

Since m can vary between $1 \le m \le n$, the total number of distinct biological function (and therefore different receptor combinations) that can be obtained will be:

$$f_n = \sum_{m=1}^{n} N_m = 2^n - 1$$

n=total number of different P2 receptor subtypes so far cloned from mammalian species. m=number of different P2

receptor subtypes present in a set (combination) of receptor subtypes. N_m=number of combinations of m receptors from the total number n of distinct P2 subtypes. f_n=total number of biological tasks generated by n distinct P2 receptor subtypes.

In this way, from n=7 P2X receptors we obtain 127 distinct functions; from n=8 P2Y receptors, we obtain 255 distinct functions, independent of the number of times in which the same receptor subtype appears in a combination. If we assume that P2X and P2Y receptors are mutually exclusive, that is, they can never appear simultaneously in the same combination, although coexisting in separate combinations in the same given cell, then the total number of possible combinations corresponding to distinct biological tasks will be 127+255=382.

Experimental evidence already supports that both P2X and P2Y receptors indeed form hetero-oligomeric complexes (or combinations), whereas hybrid P2X–P2Y receptor associations are not established yet. In the potential case in which hybrid associations will be demonstrated, the next challenge for scientists in the P2 receptor field will be to desperately seek for the 32767 distinct biological tasks sustained by n=15 total P2 receptors, as predicted by the combinatorial calculation.

At this point, we should highlight that the non combinatorial calculation of tasks sustained by each single receptor subtype would have generated only 7+8=15 different functions (instead of 382), or would have required 382 different receptors (instead of 15), in order to carry out all the same functions. Therefore, the combinatorial web condition is clearly a huge "economical advantage" for the cell, and is in turn sustained by a process of "fine-tuning", a mechanism endorsing the dynamical nature of all biological reactions. Considering that there is not yet a generally accepted definition of fine-tuning and that the principle of fine-tuning is an emerging concept in the biological sciences, its potential use in the arena of P2 receptors constitutes a highly innovative prospect and a potentially fruitful vantage point from which to view the P2 receptors.

Obvious questions arise. For instance, do the structural, pharmacological properties and molecular interactions of P2 receptors show any proof of fine-tuning? What is the evidence for fine-tuning in purinergic mechanisms? When we consider that, in order to gain the 7 cloned P2X and 8 P2Y receptors, reaching up to 99% inter-species homology and 51–17% intra-species homology, all the potential modifications of amino acid sequences must be genetically selected but also fine-tuned. This does not necessarily exclude the participation of stochastic processes, and does not necessarily mean an optimisation of the adopted solutions. Furthermore, the potencies of agonists for the various P2 receptor subtypes, which vary enormously with EC_{50} stretching from 0.1 nM (2-MeSADP on $hP2Y_{12}$ receptor) to 200 μM (β,γ-meATP on $hP2Y_{11}$ receptor subtype), strictly depend on the specie, tissue, cell type and receptor subunit composition. This proves again a clear mechanism of fine-tuning which translates the amino acid differences into correspondingly more complex molecular and biological diversities. Finally, in order to generate the heterogeneous functions ranging from neurotransmission, neurodegeneration, contraction, chemosensory signaling, immune responses, embryonic development and so on, the recruitment of selected P2 receptor subtypes on different cell phenotypes (neurons, glia, smooth and cardiac muscle, fibroblast, hepatocytes, erythrocytes, lymphocytes, platelets, spermatozoa, ovarian cells) must necessarily be driven by a process of combinatorial selection and fine-tuning. Indeed, the presence of more than one P2 receptor subtypes on a given cell, with consequent pharmacological and structural complexity, cannot otherwise be justified simply by a stochastic mechanism or by biological redundancy. The fine-tuning combines instead both genetic and epigenetic determinism resulting in the numerous physiopathological modifications of the extracellular environment. For instance, it is already known that numerous adjustments of the extracellular CNS environment directly affect P2 receptors and these comprise acute injury (hypoxia, ischemia, mechanical stress, axotomy) and chronic conditions such as pain, epilepsy, toxic ethanol and amphetamine, retinal diseases, Alzheimer's and Parkinson's diseases (Koles et al., 2005; Franke & Illes, 2006). It is therefore not by chance that the localization of the $P2X_7$ receptor on microglia, astrocytes and neurons, increases following ischemia in the cortical tissue surrounding the area of necrosis in spontaneously hypertensive rats (Franke et al., 2004b). Similarly, it increases on microglia in areas distal from the neuronal damage induced by middle cerebral artery occlusion (Melani et al., in press), pointing to a dynamic and fine-tuned role for the $P2X_7$ receptor in the pathophysiology of cerebral ischemia in vivo. In addition, whole-cell currents mediated by homomeric $P2X_2$ receptors, but not homomeric $P2X_3$ or heteromeric $P2X_{2/3}$ receptors, are reduced following an acute hypoxic challenge, possibly due to receptor down-regulation (Mason et al., 2004). Further altering the constitutive equilibrium of P2 proteins by epigenetic determinism, toxic extracellular ATP/UTP concentrations (Amadio et al., 2002; Cavaliere et al., 2005) or hypoxic-hypoglycemic conditions (Cavaliere et al., 2002, 2003, 2004a) are known to modulate the cellular expression of several different P2X and P2Y receptors. Cerebellar axotomy upregulates $P2X_1$ and $P2X_2$ receptor subtypes in rat precerebellar nuclei (Florenzano et al., 2002); microglia activation in mouse and rat brain upregulates $P2Y_6$ and $P2Y_{12}$ receptors and downregulates $P2X_7$ receptors, without affecting $P2Y_1$, $P2Y_2$ or $P2Y_4$ receptors (Bianco et al., 2005). Finally, in chronic heart failure the $P2X_6$ receptor is the only receptor subtype upregulated among the fifteen P2 receptor subtypes expressed in the heart of human patients (Banfi et al., 2005). All these are biological examples of fine-tuning, the only dynamic way to approach and integrate such a great P2 receptor sophistication.

6. Concluding remarks

We propose here the new model of "combinatorial receptor web and fine-tuning" to unravel the complexity of P2 receptors, with the goal of enquiring what ultimately distinguishes this receptor intricacy. Nevertheless, this same intricacy is the most amazing feature of P2 receptors, because of the way in which fundamental building blocks,

the P2 proteins, are recruited in an ordered way on a single cell, with even more complexity reached during physiopathological conditions. We do hope that additional information on P2 receptors and a more synergistic interaction between theory and experimental work will soon provide answers to our queries.

Acknowledgments

We are most grateful to Prof. Giorgio Bernardi for encouraging our work, to Dr. Fabio Cavaliere, Dr. Fabrizio Vacca for key experiments which inspired this review, and to Dr. Gillian E. Knight for her excellent editorial skills. The present work was supported in part by Cofinanziamenti MIUR.

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