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Review

Cytolytic P2X purinoceptors

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Abstract

Anedoctal evidence accumulated over almost 20 years has shown that many different cell types are killed by sustained exposure to high concentrations of extracellular ATP. The plasma membrane receptors involved have been pharmacologically characterized and cloned during the last 3 years, and named purinergic P2X. P2X receptors share an intriguing structural relatedness with *Caenorhabditis elegans* degenerins and mammalian amiloride-sensitive Na channels (ENaCs). Depending on the ATP dose, length of stimulation and receptor subtype, P2X receptor stimulation may cause necrosis or apoptosis. The intracellular pathways activated are poorly known, but the perturbation in intracellular ion homeostasis clearly plays a major role. ICE proteases (caspases) are also triggered, nonetheless their activation is not requested for ATP-dependent cell death. The physiological meaning of P2X receptor-dependent cytotoxicity is not understood, but an involvement in immune-mediated reactions is postulated.

Keywords: ATP; purinergic receptors; cell death; cytotocity; inflammation; apoptosis

Abbreviations: ENaC, amiloride-sensitive NA^+ channel; ICE, Interleukin 1-β. converting enzyme; Kir, inward rectifier K^+ channel; mscL, *Escherichia coli* mechanosensitive channel; CNS, central nervous system; LAK, lymphokine-activated killer cells; LPS, bacterial lipopolysaccharide; APC, antigen-presenting cell; SEB, staphylococcal enterotoxin B.

P2X receptors: what are they?

P2X receptors are plasma membrane ligand-gated ion channels activated by ATP (Abbracchio and Burnstock, 1994; Buell *et al*, 1996a). They belong to the growing family of receptors for extracellular nucleotides whose other members are the P2Y receptors (Fredholm *et al*, 1994; Brake and Julius, 1996). For this reason, P2X and P2Y receptors are collectively named P2 purinergic receptors, while plasma membrane receptors that bind adenosine are referred to as purinergic P1 (Burnstock and Kennedy, 1985). According to current classification of plasma membrane receptors for neurotransmitters, P2X and P2Y receptors are also often referred to as ionotropic and metabotropic, respectively (Abbracchio and Burnstock, 1994). Early evidence for the presence of ATP-ligated receptors goes back to observation by Holton and coworkers on vasomotor responses evoked by antidromic stimulation of the rabbit ear (Holton and Perry, 1951; Holton and Holton, 1954) and of Burnstock and colleagues on the contracturant effect of ATP applied to vascular smooth muscle (Burnstock *et al*, 1972). Now it is generally accepted that extracellular nucleotides play a major role in cell to cell communication (Brake and Julius, 1996). It was initially thought that P2X receptor/channels were only expressed by excitable cells, but it is clear that they are also present in many other cells of widely different origin (Buell *et al*, 1996a). Recently, their identification in mononuclear phagocytes and lymphocytes has attracted great interest in view of a possible involvement in immune modulation (Di Virgilio *et al*, 1996a,b).

Molecular structure of P2X receptors

The first cloning of P2X receptors was reported in 1994 by two different groups. Valera *et al* (1994) isolated a cDNA encoding an ATP-gated channel from smooth muscle of rat vas deferens, while Brake *et al* (1994) isolated a cDNA of another ATP-gated channel from the rat pheochromocytoma cell line PC12. The smooth muscle clone was named $P2X_1$, while the PC12 clone was named $P2X_2$. Following these initial reports, several clones for other P2X receptors have been isolated from rat and human cells, amounting now to a total number of seven (Buell *et al*, 1996a). Determination of the aminoacid sequence has unveiled a surprising structural similarity of P2X channels with other families of ion channels such as degenerins of *Caenorhabditis elegans* (Deg-1, Mec-4 and Mec-10), the epithelial sodium channels of mammalian cells (ENaCs), the inward-rectifier potassium channel (Kir), and the mechanosensitive channel of *E. coli* (mscL) (Corey and Garcia-Anoveros, 1996; North, 1996) (Figure 1). All these families of ion channels share the same structural motif: two putative membrane spanning domains with both the N and C

termini on the cytoplasmic side and a bulky extracellular domain (Figure 2). Kir is an exception, as this channel has a smaller extracellular domain that forms a loop that goes back half way across the plasma membrane.

Size of P2X channels ranges from 379 aminoacids for $P2X_6$ (Collo *et al*, 1996; Soto *et al*, 1996b) to 595 for $P2X_7$, the largest member of the family (Suprenant *et al*, 1996; Rassendren *et al*, 1997). Overall, P2X channels share little sequence identity. The sharing of sequence identity is higher in the N-terminus and in the putative extracellular domain, and very low in the C terminus. Furthermore, ten cysteine residues are conserved in the extracellular region, suggesting that establishment of S-S bonds may cause a highly convoluted tertiary structure. The only known agonist for these channels is ATP, but quite intriguingly their putative extracellular domain shows no obvious ATP-binding sequences. It has been suggested that, in analogy to a previous proposal for the catalytic site of ATPases, parallel β sheet structures that are likely to be present in the ectodomain of P2X receptors may generate a binding site for ATP, but this is still to be proven (Buell *et al*, 1996a). No other soluble physiological activator of P2X channels has been so far discovered, nor is it known whether the bulky ecto-domain can interact with structural components of the extracellular matrix, as shown for unc-105 of *C. elegans* (Liu *et al*, 1996), thus endowing P2X channels with the ability to respond to mechanostimulation. It may be of interest that Zn^{2+}, a cation that is abundant in the central nervous system (CNS), highly potentiates current responses of some P2X channels by shifting the ATP dose-dependency to lower concentrations (Li *et al*, 1993). This is reminiscent of the effect of Zn^{2+} on NMDA receptor activity (Hollman *et al*, 1993), although in this case increase in potency is due to enhancement of maximal response without altering the agonist dose-response curve. Potentiation by Zn^{2+} could be due to allosteric modulation or to interaction with ATP, but this is not currently known.

As pointed out above, the COOH tail is the less conserved domain among the seven members of P2X subfamily. It is not known if this may determine the functional behaviour of the different P2X receptors/channels, with one relevant exception, $P2X_7$. The C-terminal in this receptor is much longer than in other P2X channels (for example, 28 aminoacids for $P2X_4$, and 118 for $P2X_2$ *versus* 240 for $P2X_7$). Truncation of $P2X_7$ C-terminal at position 418 drastically modifies the permeability properties, changing this receptor from a non-selective pore into a cation selective channel (see below) (Suprenant *et al*, 1996). However, while the C-terminal tail of $P2X_7$ appears to be necessary for the pore-forming activity of $P2X_7$, it does not seem to be sufficient by itself to confer the pore-forming activity, as chimeric $P2X_2$ receptors in which the native C-tail was replaced with the $P2X_7$ C-tail, do not form pores (Anne marie Surprenant, personal communication).

While there are few doubts that the functional channel must consist of multiple P2X subunits, it is not known whether the native channel is formed by homo or hetero polymerization, nor do we have hints as to subunit stoichiometry. Only for $P2X_2$ and $P2X_3$ found in sensory neurons has it been shown that the native receptor is very likely formed by heteromultimerization of the two different subunits (Chen *et al*, 1995; Lewis *et al*, 1995), maybe in a pentameric structure.

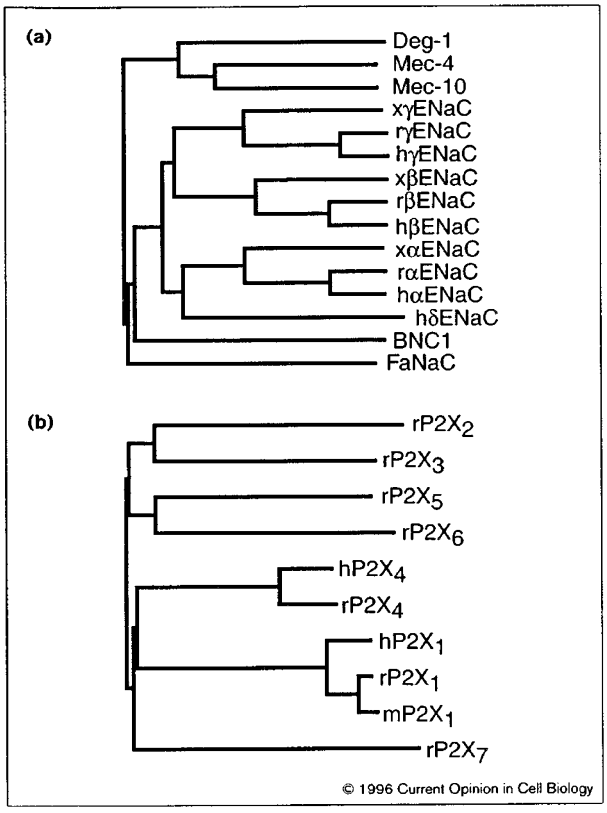

Figure 1 Dendrograms showing the structural relatedness within the two proposed subfamilies of plasma membrane channel subunits with two hydrophobic domains. (**a**) Relatedness within the subfamily of *C. elegans* degenerins (Deg-1, Mec-4 and Mec-10), mammalian epithelial Na channels (ENaC), human brain Na channel (BNC1) and *Helix aspersa* FMRFamide-gated channel (FaNaC). (**b**) Relatedness within the family of P2X channels. h, human; r, rat; m, mouse; x, *Xenopus* (reproduced from North, 1996, with permission)

Functional properties of P2X receptors

P2X receptors form cation selective channels. In patch clamp experiments, ATP EC_{50} for activation of cloned rat P2X receptors ranges from 0.7 μM ($P2X_3$) to 115 μM ($P2X_7$). At least 10-fold higher ATP concentration is needed to activate the cloned human $P2X_7$ receptor. High agonist concentrations have also been reported for activation of native P2X receptors in macrophages, fibroblasts and mast cells (see Steinberg and Silverstein, 1987; Buisman *et al*, 1988; Tatham and Lindau, 1990; Pizzo *et al*, 1992). Although permeability properties are not fully defined, there is substantial agreement that Na^+ and K^+ are equipermeant while Cs^+ is less permeant (Valera *et al*, 1994; Brake *et al*, 1994; Soto *et al*, 1996a). In general, Ca^{2+} is also very permeant ($P_{Ca}/P_{Na} \approx 4$) (Buell *et al*, 1996a), but there is some controversy on this issue in the previous literature, probably due to cell type differences (Benham and Tsien, 1987; Friel and Bean, 1988;

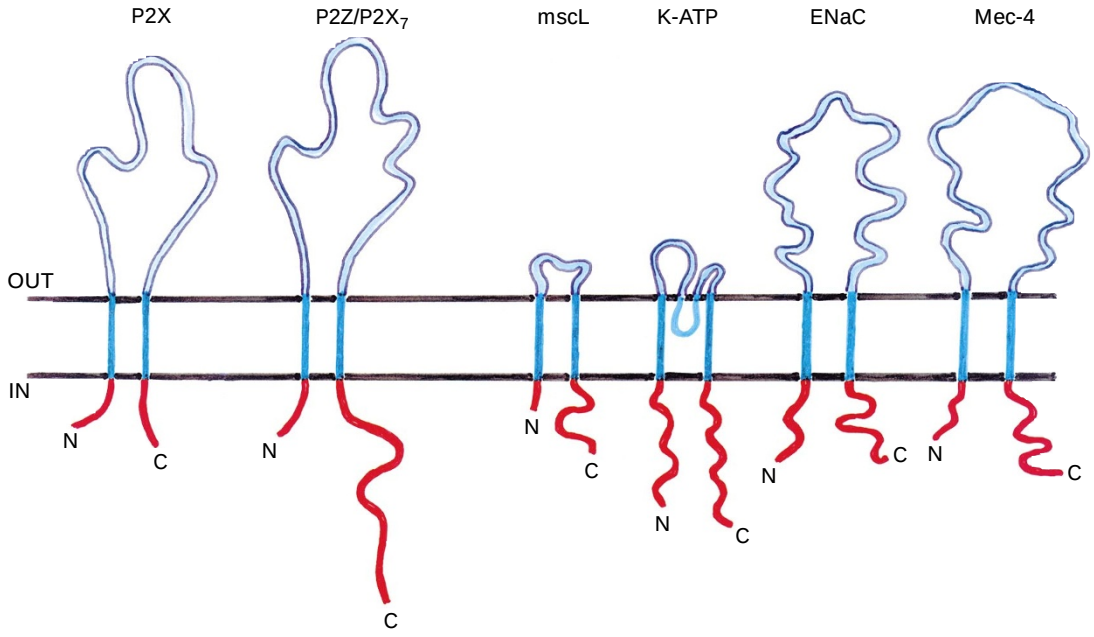

Figure 2 Schematic drawing of different types of channels with two hydrophobic domains. This drawing shows the general architecture of ATP-gated channel subunits (P2X), the peculiar P2Z/$P2X_7$ subunit (note the extended COOH tail), the mechanosensitive channel of *E. coli* (mscL), the ATP/ADP-gated inward rectifier K^+ channel (K-ATP), the mammalian epithelial amiloride-sensitive Na channel (ENaC), the *C. elegans* mechanosensitive channel (Mec-4). Light blue, ectodomain; green, membrane-spanning regions; red, cytoplasmic domains

Bean *et al*, 1990). $P2X_7$ has a completely different and unique behaviour. While brief applications of ATP evoke inward currents carried by cations, sustained stimulation (or repetitive applications) induce the appearance of a large non-selective pore (Surprenant *et al*, 1996; Rassendren *et al*, 1997). This pore allows transmembrane fluxes of choline, methylglucamine, propidium iodide, YO-PRO and lucifer yellow. This behaviour closely mimicks that of the native ATP-gated channel, preliminary named P2Z, described in fibroblasts (Rozengurt *et al*, 1977; Makan and Heppel, 1978; Pizzo *et al*, 1992), mast cells (Cockcroft and Gomperts, 1979), macrophages (Steinberg and Silverstein, 1987; Steinberg *et al*, 1987; Di Virgilio *et al*, 1988) and other cells (Kitagawa and Akamatsu, 1986). Activation of this receptor by ATP induces uptake of extracellular tracer molecules such as lucifer yellow and even the Ca^{2+} chelator fura-2 in its acidic form. The pharmacology of $P2X_7$ and the native P2Z receptor is very similar: both appear to be activated by ATP^{4-}, rather than by Ca/Mg-ATP, and for both receptors benzoylbenzoylATP is about tenfold more active than ATP (Steinberg and Silverstein, 1987; Murgia *et al*, 1992a; Suprenant *et al*, 1996; Rassendren *et al*, 1997). In our laboratory we have selected several cell clones from J774 macrophages and N13 or N9 microglial cells that functionally either lack or hyperexpress the P2Z receptor (Murgia *et al*, 1992a; Chiozzi *et al*, 1996; Ferrari *et al*, 1996). P2Z-less and P2Z-high clones are also negative or strongly positive, respectively, to *in situ* hybridization with a specific $P2X_7$ probe or to a polyclonal antibody directed against the COOH tail of $P2X_7$ (Chiozzi *et al*, 1997; Ferrari *et al*, 1997a). This is strong evidence in favour of P2Z being formed by the assembly of $P2X_7$ subunits, although it is not clear whether other P2X subunits ($P2X_1$?) may also participate to the channel phenotypically known as P2Z. Macrophages and microglial cells may express other ATP-gated receptors in addition to P2Z/$P2X_7$. This is evident from the observation that macrophage and microglial cell clones negative for P2Z/$P2X_7$ receptor expression are transiently depolarized by ATP, as if they expressed an inactivating ATP receptor (Ferrari *et al*, 1996). Furthermore, $P2X_1$ expression has been detected in HL60 and THP-1 cells at various levels of differentiation (Buell *et al*, 1996c; Dubyak *et al*, 1996).

The most striking property of P2Z/$P2X_7$ is the ability to mediate a reversible permeabilization of the plasma membrane, provided the ATP pulse is of short duration (10–15 min) (Rozengurt *et al*, 1977; Gomperts and Fernandez, 1985; Steinberg and Silverstein, 1987; Di Virgilio and Steinberg, 1993). More sustained stimulations cause an irreversible cell damage that eventually leads to cell death (Kitagawa *et al*, 1988; Nagelkerke *et al*, 1989; Blanchard *et al*, 1991; Murgia *et al*, 1992b; Falzoni *et al*, 1995; Modderman *et al*, 1994; Vijweide *et al*, 1995; Zoeteweij *et al*, 1996).

P2X receptors and cell death

ATP-gated plasma membrane channels were firstly described in fibroblasts and mast cells about 20 years ago. Yet for a long time they were considered little more than a curiosity and no effort was made towards the understanding of their role in cell physiology. In the second half of the 80s, a few laboratories undertook a systematic investigation of these receptor/channels and showed that sustained stimulation of certain

cells and cell lines (melanoma and mouse fibroblast cells, J774 mouse macrophages, primary mouse and human lymphocytes, mouse lymphoma and mastocytoma cells) with extracellular ATP caused cell death (Steinberg and Silverstein, 1987; Kitagawa *et al*, 1988; Di Virgilio *et al*, 1989; Wiley and Dubyak, 1989; Zanovello *et al*, 1990). Our group also showed that cell death could be due to either colloido-osmotic lysis or apoptosis depending on the cell type and length of stimulation (Zanovello *et al*, 1990; Murgia *et al*, 1992a). These early demonstrations were extensively confirmed by several other laboratories both in primary cell cultures and in cell lines (Filippini *et al*, 1990a; Zheng *et al*, 1991; Blanchard *et al*, 1991, 1995; Spranzi *et al*, 1993; Zoetewij *et al*, 1996; Macino *et al*, 1996; Correale *et al*, 1997), and eventually validated in HEK293 cells stably transfected with the recombinant $P2X_7$ receptor (Suprenant *et al*, 1996; Ferrari *et al*, 1997a). Early studies also showed that some cell types, such as cytotoxic T lymphocytes (CTLs) and lymphokine-activated killer (LAK) cells were inherently refractory to ATP stimulation (Di Virgilio *et al*, 1989; Zanovello *et al*, 1990; Filippini *et al*, 1990a). Although protection could be afforded in theory by a high expression of plasma membrane ecto-ATPases, there are now few doubts that the main reason resides in the lack of expression of P2X receptors in these cells. Along this line, we showed that CTLs and LAK cells remained resistant even in the virtual absence of extracellular divalent cations (Di Virgilio *et al*, 1989), a condition that prevents enzymatic activity of ATPases. In addition, CTLs do not express an ectoATPase activity significantly greater than that measured in ATP-sensitive cells. All these observations suggested that susceptibility to ATP-mediated cytotoxicity was conferred by expression of a plasma membrane ATP-gated channel, and refractorines depended on the absence of such channel. Following studies demonstrated that ATP was cytotoxic not only to cells that express the typical 'permeabilizing' P2Z receptor, such as macrophages, but also to those cells in which this nucleotide activates a cation-selective channel, with no evidence of permeabilization to larger hydrophylic solutes (Pizzo *et al*, 1991; Ferrari *et al*, 1994). This lead us to suggest that plasma membrane ATP-gated channels may form a new family of ion channels with different permeability and ion selectivity, but linked by the common property of causing cell death under conditions of sustained stimulation (Murgia *et al*, 1992b). Recent data have confirmed that immune cells susceptible to ATP-mediated cytotoxicity express either $P2X_1$ or $P2X_7$ or maybe both (Buell *et al*, 1996c; Chvatchko *et al*, 1996; Dubyak *et al*, 1996). There has been some discussion in the past about the possibility that some cytolytic effect could be mediated via P2Y receptors. However, this can be now safely excluded because ATP-resistant macrophages and microglial cell clones do express P2Y receptors to a level indistinguishable from that found in sensitive cells.

It was not completely unexpected that prolonged activation of P2X channels could cause necrotic lysis as several bacterial toxins (Fussle *et al*, 1981) or immune soluble factors (i.e. complement or perforins) (Young *et al*, 1986; Henkart, 1985) are known to lyse cells by causing formation of plasma membrane pores. Furthermore, as pointed out by Pizzo *et al* (1991), ATP receptors share intriguing functional similarities with receptors for excitatory aminoacids in central neurons. These latter receptors also gate channels that cause Ca^{2+} influx in the absence of Ca^{2+} mobilization from stores, trigger depolarization of plasma membrane and are involved in cell death (excitotoxicity). On the contrary, it was somewhat unexpected that P2X receptor activation could also cause apoptosis.

The first indication that P2X receptors could be involved in apoptosis came from experiments performed in mouse P815 cells. In these cells, ATP was shown to cause a clear DNA fragmentation, indistinguishable from that found as a consequence of co-incubation with lymphokine-activated killer (LAK) cells (Zanovello *et al*, 1990), well known cytotoxic effectors that kill target cells by apoptosis. DNA fragmentation was specifically triggered by ATP, very rapid and associated with the induction of transmembrane ion fluxes. The apoptotic effect of ATP was soon confirmed by Zheng *et al* (1991) in mouse thymocytes. These authors performed a thorough morphological investigation showing that ATP not only caused DNA fragmentation, but also membrane blebbing, cell shrinkage, nuclear condensation and apoptotic bodies formation. Interest in the possible involvement of P2X receptors in apoptosis was rekindled by the first reports of the aminoacid sequence and tentative membrane topology of P2X receptors from rat smooth muscle (Valera *et al*, 1994) and rat pheochromocytoma (PC12) (Brake *et al*, 1994) cells. It was immediately apparent that $P2X_1$ was nothing but the product of the RP-2 gene, which had previously been shown to be expressed during the early phases of apoptosis in rat thymocytes (Owen *et al*, 1991). Further support for a role of $P2X_1$ has recently come from the demonstration that mouse thymocytes exhibit an increase of $P2X_1$ mRNA content after *in vitro* dexamethasone treatment and *in vivo* challenge with the superantigen staphylococcal enterotoxin B (SEB) (Chvatchko *et al*, 1996 but see also Jiang *et al*, 1996, for an opposite view).

The ATP degradation product adenosine has also been shown to cause apoptosis in several cell types via activation of a purinergic P1 receptor of the A_1 or A_3 subtype (see Abbracchio, 1996, for a recent review).

Human monocyte-derived macrophages can undergo ATP-dependent apoptosis, especially if stimulated in a pulsed rather than continuous fashion (Molloy *et al*, 1994). In the case of a sustained stimulation, the most likely outcome is cell death by necrosis. Other factors may also affect the final outcome (i.e. necrosis *versus* apoptosis) as primary human and mouse macrophages appear to be more easily induced to apoptosis by ATP, while some tumor cell lines, such as the J774 mouse macrophages, in our experience are more prone to undergo necrosis (Murgia *et al*, 1992a). Susceptibility of macrophages to ATP-dependent death may have significant practical applications as Molloy *et al* (1994) have shown that stimulation with ATP of macrophages infected with the intracellular organism bacillus Calmette Guerin (BCG), not only caused apoptosis of the macrophages but also killed the intracellular organism. On the contrary, macrophage killing with other cytotoxic agents, e.g. hydrogen peroxide,

released viable microrganisms. This observation has been recently confirmed with BCG and also extended to a virulent strain of *Mycobacterium tuberculosis* (V. Vishwanath, S. Falzoni, P. Chiozzi and F. Di Virgilio, unpublished observations; G. Mancino, G. Barbolini, F. Di Virgilio and V. Colizzi, manuscript in preparation).

Mechanism of P2X receptor-mediated cytotoxicity

Cell death appears to be due to the prolonged opening of P2X channels in the presence of the agonist, resulting in the activation of a non desensitizing channel. Although most of P2X receptors (i.e. $P2X_2$ and $P2X_{4\text{-}7}$, but quite intriguingly not $P2X_1$) fulfil this criterion, any extrapolation from cloned to native receptors is dangerous, as in many cases electrophysiological behaviour of transfected P2X receptors is affected by the recipient cell (e.g. P2X4 expressed in HEK293 cells *versus* oocytes) (Buell *et al*, 1996c; Seguela *et al*, 1996; Soto *et al*, 1996a). Early cellular events following P2X receptor activation include Ca^{2+} and Na^+ influx, K^+ efflux, plasma membrane depolarization, swelling and disaggregation of the cytoskeletal network. In macrophages, extensive formation of phase lucent vesicles is also observed. How these changes contribute to cell death is not well understood, however it is clear that the inbalance in ion homeostasis plays a crucial role. In the presence of physiological concentration of monovalent cations, Ca^{2+} is not needed for ATP-dependent cytotoxicity. Replacement of extracellular Na^+ and K^+ with isoosmolar Ca^{2+}-free sucrose greatly delays cell death, that however is rapidly triggered by re-addition of Ca^{2+} (Pizzo *et al*, 1991; Murgia *et al*, 1992a). Thus it seems that while Ca^{2+} is not needed for ATP-mediated cytotoxicity in standard Na^+ and K^+-containing buffers, it is however capable of restoring the ATP cytotoxic effect when added to a sucrose medium that by itself does not sustain cytotoxicity. Whether Ca^{2+}-dependent and independent ATP-mediated cytotoxicity involves different intracellular pathways has not been investigated.

Participation of other intracellular second messengers has not been explored, but for a recent report showing that tyrosin kinases and phosphatases may be involved (Bronte *et al*, 1996).

In macrophages and microglial cells, ATP triggers production of mature interleukin-1β via activation of interleukin-1β-converting enzyme (ICE) (Perregaux and Gabel, 1994; Perregaux *et al*, 1996; Ferrari *et al*, 1996, 1997b), a member of the growing family of cystein proteases (caspases) that mediate apoptosis (Alnemri *et al*, 1996; Nagata, 1997). Experiments performed in our laboratory with the specific cell permeant substrate YVAD have further confirmed that extracellular ATP triggers ICE activation (J. Sanz and F. Di Virgilio, manuscript in preparation). However, it seems also clear that this pathway is not needed for ATP-dependent cytotoxicity, as YVAD-inhibited macrophages are still susceptible to ATP-dependent apoptosis (Nett-Fiordalisi *et al*, 1995). In addition, also macrophages from knock-out mice for ICE, which are unable to produce IL-1β in response to ATP, undergo ATP-dependent apoptosis (Li *et al*, 1995). This is in agreement with other reports showing that ICE activation is not an obligatory step in the apoptotic pathway (Nagata, 1997).

The issue of whether stimulation of the $P2X_7$/P2Z receptor is always associated to cell death, is controversial. In general, the outcome is very dependent on the target cell type, presumably reflecting the density of $P2X_7$/P2Z receptor expression. There are also species differences, as the human $P2X_7$ has a lower affinity than the rat counterpart (Rassendren *et al*, 1997), and likewise higher nucleotide concentrations are required to induce permeabilization of human than mouse macrophages (Hickman *et al*, 1994; Falzoni *et al*, 1995). This may explain why ATP is a good apoptotic stimulus for human but not J774 mouse macrophages, as these latter cells, being more sensitive, are more prone to undergo fast ATP-mediated necrosis rather than apoptosis. In the mouse macrophage cell line J774, a short (less than 15 min) activation of the $P2X_7$/P2Z receptor causes a fully reversible permeabilization. These cells, after the short ATP pulse, resume their pre-pulse morphology, spread on the substrate again and start to divide. The ability to fully recover physiological functions after permeabilization has been the basis for the selection and establishment of J774 cell clones that express a high level of $P2X_7$/P2Z receptor (Chiozzi *et al*, 1996, 1997).

P2X receptors and cytotoxicity: a laboratory curiosity or a physiologically relevant phenomenon?

The physiological meaning of ATP-mediated cytotoxicity is an open question. There are few doubts that ATP is released via non lytic pathways by many cells. Central (White *et al*, 1978) and peripheral (von Kugelgen and Starke, 1991; Todorov *et al*, 1997) neurons and platelets (Meyers *et al*, 1982; Colman *et al*, 1990) release ATP by exocytotic secretion; epithelial and endothelial cells also release ATP (Pearson and Gordon, 1979; Reisin *et al*, 1994), maybe via plasma membrane transporters belonging to the family of ABC (ATP-binding cassette) proteins (Abraham *et al*, 1993, 1996; Reisin *et al*, 1994; Schwiebert *et al*, 1995); T lymphocytes (Filippini *et al*, 1990b) and macrophages (Ferrari *et al*, 1997c) also secrete ATP in the pericellular space via a yet to be identified mechanism. Furthermore, being the average cytoplasmic ATP concentration in the range of 5–10 mM, any agent that causes acute plasma membrane damage is also expected to release ATP. Thus, it is likely that under physiological and pathological conditions significant ATP concentrations can accumulate in the extracellular space, may be at sites of close cell-to-cell contact, where in a protected compartment ATP will not easily dilute into the interstitial fluid. Obviously, the extracellular ATP concentration is affected not only by ATP release but also by ATP hydrolysis by ubiquitous ecto-ATPases (Zimmermann, 1996). Recent experiments by Robson and co-workers (1997) show that during an inflammatory reaction the ATP diphosphohydrolase activity is downregulated, thus providing an additional factor that contributes to the accumulation of extracellular ATP.

Our and Sitkovsky's group originally proposed that extracellular ATP could participate in cytotoxic reactions in the immune system (Di Virgilio *et al*, 1989, 1990; Filippini

et al, 1990a). Admittedly, there was no direct proof supporting this hypothesis, but rather a number of circumstantial observations such as the Ca^{2+} independency of ATP-mediated cytotoxicity, the apoptotic mechanism, the strong synergism with TNF (Bronte *et al*, 1993), the remarkable refractoriness to ATP of cytotoxic T cells and of Lymphokine Activated Killer (LAK) cells (Di Virgilio *et al*, 1990; Filippini *et al*., 1990a). This hypothesis was probably too naive as originally postulated. Nonetheless, convergent reports from many different laboratories point to an important role for ATP and purinergic P2X receptors in cytotoxic events during the immune and inflammatory reactions.

Blanchard *et al* (1995) have recently proposed that ATP receptors could be involved in the elimination of antigen-presenting cells by $CD4^+$ and $CD8^+$ T lymphocytes. This observation is potentially of great interest as killing of antigen-presenting cells (APCs) is known to participate in the down regulation of the immune response. Expression of the P2Z/$P2X_7$ receptor is up-regulated by IFN-γ (Blanchard *et al*, 1991; Falzoni *et al*, 1995; Humphreys and Dubyak, 1996), a cytokine that is also well known for increasing expression of MHC-II molecules and therefore antigen-presenting activity of APCs (Farrar and Schreiber, 1993). Thus, it seems that IFN-γ-stimulated macrophages on one hand increase their ability to stimulate the immune response, and on the other express a safe-guard device (the P2Z/$P2X_7$ receptor) that act as a feed-back, inhibitory mechanism. The powerful inflammatory stimulus bacterial lipopolysaccharide (LPS) can also upregulate P2Z/$P2X_7$ receptor expression in THP-1 macrophages (Humphreys and Dubyak, 1996).

Likely occurrence of significant accumulation of extracellular ATP at sites of immune and inflammatory reactions, upregulation of the prototypical cytotoxic receptor (P2Z/$P2X_7$) by inflammatory cytokines and bacterial products and increased susceptibility to ATP of macrophages as compared to blood monocytes, suggest that P2X purinergic receptors play an important role in the defense mechanisms of the body (Di Virgilio, 1995). In certain conditions (i.e. in the presence of extensive tissue damage, strong stimulation of active ATP release or powerful inhibition of plasma membrane ecto-ATPases (see Robson *et al*, 1997), or maybe under the cooperative effect of all these factors together), sufficient ATP may accumulate in the pericellular space to trigger P2Z/$P2X_7$ receptor activation and thus cell death. P2Z/$P2X_7$-mediated cell death should not be necessarily accidental, but in some cases might be the result of a coordinated activation of immune cells, aimed for example at the killing of antigen presenting cells (APCs) during the physiological process of antigen presentation, or at the elimination of phagocytes parasitized by pathogens that survive phagocytosis ('Samson shall die with all the Philistines!') (Di Virgilio, 1995).

We have proposed that the 'suicide' P2Z/$P2X_7$ receptor could also have a role in spontaneous cell death, such as that occurring in cell cultures. Chiozzi *et al* (1996) have shown that J774 macrophages *in vitro* undergo a basal rate of cell death that is significantly increased upon reaching confluence. The extent of cell death is much higher in those J774 macrophage clones that hyper express the P2Z/$P2X_7$ receptor and virtually nil in the clones that were selected for lack of P2Z/$P2X_7$. In addition, incubation in the presence of ATP-consuming enzymes, such as apyrase or hexokinase, or pretreatment with oxidized ATP, an inhibitor of the P2Z/$P2X_7$ receptor, greatly reduced cell death. Our interpretation of these experiments is that macrophages *in vitro* are continuously exposed to an autocrine/paracrine stimulation by ATP, that causes a basal activation of the P2Z/$P2X_7$ receptor which in turn might trigger a cytotoxic response when a given threshold is reached.

Cytotoxicity may be just one of many functions. At low levels of stimulation (i.e. low extracellular ATP concentration), P2X receptors primarily co-operate in cell activation (e.g. cytokine release or lysosomal content release), or even participate in cell fusion events (see Falzoni *et al*, 1995 and Chiozzi *et al*, 1997).

$P2X_1$, the other P2X receptor for which a role in cell death has been proposed, could be involved in thymic selection. In experiments reported by Chvatchko *et al* (1996), $P2X_1$ receptor expression was upregulated in thymocytes by apoptotic stimuli such as dexamethasone and SEB, a superantigen that ligates the T cell receptor. Furthermore, exogenous ATP caused apoptosis that was prevented by apyrase, an enzyme that degrades ATP. Since these effects were restricted to thymocytes (peripheral T lymphocytes appear not to express $P2X_1$), the authors propose that this ATP-gated channel could be involved in clonal deletion in the thymus.

Conclusions and future perspectives

Cloning and sequencing and functional characterization of ATP-gated ion channels (P2X receptors) has disclosed an intriguing new family of plasma membrane channels that share structural homology with other ion channels (degenerins) known to be involved in cell death in *C. elegans*. Amiloride-sensitive Na channels related to degenerins (MDEGs for mammalian degenerins) have been recently cloned from human brain. It appears that two membrane-spanning channels may form a new superfamily of molecules endowed with the ability to mediate, at least *in vitro*, a death signal. Several questions await an answer: is there any evidence that cytotoxic activity is also present *in vivo*? Is extracellular ATP the physiological ligand, or have we so far missed the actual activatory counterreceptor (e.g. a matrix or soluble protein)? What is the mechanism of subunit assembly? Do these receptors interact with other intracellular proteins?

We think that clarification of these, and many other unsolved questions, will not only deepen our knowledge of cell physiology and metabolism but will also provide us with new therapeutic agents.

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