REVIEW ARTICLE

$P2X_7$ receptor at the heart of disease

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Abstract

Purinergic signaling is a crucial component of disease whose pathophysiological basis is now well established. This review focuses on $P2X_7$, a unique bifunctional purinoreceptor that either opens a non selective cation channel or forms a large, cytolytic pore depending on agonist application and leading to membrane blebbing and to cell death either by necrosis or apoptosis.

Activation of $P2X_7$ receptor has been shown to stimulate the release of multiple proinflammatory cytokines by activated macrophages, with the IL-1b to be the most extensively studied among them. These findings were verified by the use of knockout $P2X_7^{(-/-)}$ mice.

Update information coming from all fields of research implicate this receptor at the very heart of diseases such as rheumatoid arthritis, multiple sclerosis, depression, Alzheimer disease, and to kidney damage, in renal fibrosis and experimental nephritis.

Clinical studies are currently underway with the newly developed selective antagonists for $P2X_7$ receptor, the results of which are eagerly anticipated. These studies together with data from in-vivo experiments with the $P2X_7$ knockout mice and in-vitro experiments will shed light in this exciting area. Hippokratia 2010; 14 (3): 155-163

Key words: $P2X_7$, NLRP3 inflammasome, IL-1b ATP, purinergic signaling, purinergic receptors, review

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Whilst conducting experiments in the guinea-pig taenia coli, G. Burnstock, in the early 70s, observed the presence of a third, non adrenergic, non cholinergic neurotransmitter, and claimed it to be the extracellular Adenosine-5- Triphosphate (ATP)[1,2]. Two decades later, the discovery of purinergic receptors[3] shed light on the mechanism underlying this purinergic signaling and demonstrated that ATP exerted its multiple actions via specific purinoreceptors located in plasma membranes.

In 1994, classification and nomenclature of purinergic receptors, based on their pharmacological properties, revealed the existence of a distinct P1 adenosine receptor and P2 receptors, further divided into P2X ionotropic receptors and P2Y metabotropic G-coupled protein receptors[4-7].

Purinergic signaling was finally accepted as a crucial component of disease and was found to mediate a vast array of biological processes such as neuronal transmission, signal transduction to the cardiovascular system, mediators of exocrine and endocrine functions, and involvement in immunity, inflammation and cancer[8].

ATP and purinergic receptors

ATP is constantly generated intracellularly by mitochondrial oxidase phosphorylation and via cytolytic glycolysis and stored in the cytoplasm of cells such as platelets[9] macrophages[10], microglia[11] and activated immune cells in concentrations that reach molar range (3M).

ATP cannot be transported across lipid bilayers by simple diffusion because of its size and charge and is currently thought to exit cells either through vesicular transport or channel-mediated release like the ATP-binding cassette transporters, gap junction hemichannels, connexin hemichannels (CX), and anion channels such as: cystic fibrosis transmembrane conductance regulator (CFTR), volume sensitive outwardly rectifying (VSOR) and maxi-anion channels[12].

Dying[13] or stressed cells secondary to hypoxia, ischemia, osmotic swelling and mechanical stimulation[14,15] may release high concentrations of ATP into the pericellular space. The regulated release of ATP plays an essential role in autocrine and/or paracrine cell-to-cell signaling. As soon as ATP successfully crosses the plasma membrane borders, it is rapidly degraded by the ubiquitously extracellurly present ectonucleotidases[16]. In these compartments ATP resynthesising enzymes known as kinases, has been documented.

Purinergic receptors: classification, nomenclature, cloning

All cells express plasma membrane receptors for extracellular nucleotides called the purinergic receptors[3].

These receptors are classified as P1 and P2 receptors. P1-adenisine receptors activated by adenosine, include four cloned members; A1, A2B, A2A, and A3[17]. P2 receptors are further subdivided into P2X receptors known as ionotropic purinergic receptors, that include seven members $P2X_7$ and the P2Y metabotropic receptors activated by ATP and Adenosine Diphosphate (ADP). P2Y receptors have eight different forms[6]. The discovery of P1 adenosine receptor was first proposed by Burnstock in 1978 and later the distinction of P2 receptors in P2X and P2Y was based on their separate pharmacological properties[7].

Structure and properties of $P2X_7$ receptor

$P2X_7$ receptor is a protein that results from the homomerization of three subunits[18] and was first cloned from a rat brain library[19], then from human monocytes[20] and finally from mouse microglia cells[21]. $P2X_7$ is mainly expressed in cells of the haemopoetic lineage such as: antigen-presenting immune cells and epithelia, monocytes/macrophages, leukocytes, red cells, fibroblasts, dendritic cells, keratinocytes, astrocytes, microglia, lymphocytes, mast cells and Langerhans cells of the epidermis[20,22,24].

$P2X_7$ receptor constitutes from:

a. Two transmembrane protein domains with 472 amino acids.

b. A long extracellular loop, with 10 residues of cysteine, that may form disulphide bonds[25]. The disulphide bonds are thought to be ATP-binding sites[26] positive charged residues of lysine, 2-6 posts of glykosylation.

c. Two intracellular termini, one amino N-terminus and one carboxyl, C-terminus[19,27]. The C-terminus in $P2X_7$ is at least 120 amino acids longer than the rest of its family members[28].

Ion current properties

Brief exposure to agonist ATP leads to the opening of cation channel that permits K^+ efflux and Ca^{2+} and Na^+ influx into the cells. This causes a major disturbance to the ionic gradient across the plasma membrane allowing calcium influx into the cell, thus triggering several intracellular signaling cascades.

Recently another agonist was found to activate $P2X_7$ receptor , and this was cathelicidin (LL37) which is a potent antimicrobial peptide produced predominantly by neutrophils and epithelial cells. LL37 has been shown to activate the $P2X_7$ receptor at much lower concentrations than ATP and promotes IL-1b processing and release without causing cytotoxicity[29].

Prolonged activation of the agonist on $P2X_7$ receptor results in the formation of a large aqueous pore permeable to molecules of a molecule mass up to 900 Da. There are also rapid membrane and mitochondrial morphological changes, cytoskeletal rearrangement, and eventual cell death[30,19,31,32].

Current evidence implicates the C-terminul as a critical size for the formation of the large pore, since it interacts with 11 intracellular proteins, cytoskeletal and signal transduction proteins[33]. Deletion of the cytoplasmic tail did not affect ion channel properties but severely affected the ability to form a large pore and to induce activation of caspases[34].

Pore formation is essential for $P2X_7$ stimulated IL-1b release[35]. The mechanism of pore formation by the $P2X_7$ is a matter of debate. Some investigators believe that pore formation is due to an ATP-dependent increase in size of the $P2X_7$ channel itself whilst others believe that the pore is a separate molecular structure activated by the $P2X_7$[36, 37].

The $P2X_7$ is non-desensitizing receptor. The pore stays open as long as it is bound by its ATP ligand. Removal of the nucleotide, by rinsing or apyrase-catalyzed hydrolysis[38] causes pore closure, thus allowing reversible plasma membrane permeabilization.

The gene that encodes $P2X_7$ protein is on chromosome 12q24 and is a highly polymorphic gene. More than 260 single nucleotide polymorphisms (SNPs) have been described in the human $P2X_7$ gene, but only a few have been functionally characterized. Several studies[39-41] have identified four loss-of-function single amino acid substitutions[26,42,43]. No convincing disease associations have been demonstrated for these SNPs.

Cabrini et al group has characterized the first gain-of-function polymorphism so far identified (H155Y)[44]. This raises the obvious question whether or not some of the actions that have been associated with $P2X_7$ receptor may be due to the participation of more members of P2X family. Alternatively $P2X_7$ receptors close connection to massive release of mature IL-1b by activated macrophages, may suggest these receptors acting as danger sensors, that make the critical decision of continuing inflammation to the next level or turn off inflammatory detrimental consequences thereby preventing it from becoming chronic. The responses of $P2X_7$ to ATP and 3′-0-(4-benzoyl) benzol adenosine 5′-triphosphate (BzATP) are increased by reducing the concentration of extracellular divalent cations[30,45-50] such as zinc and copper whereas, other P2X receptors are strongly potentiated or unaffected$_7$.

IL-1beta and $P2X_7$ receptor activation

IL-1b is a master proinflammatory cytokine that exerts a broad range of inflammatory processes regulating the host response to infections, activating macrophages and neutrophils and inducing Th1 and Th2 cellular responses[51-53]. Given its detrimental role in inflammation, living organisms have developed a tight control mechanism in the release of bioactive IL-1b.

IL-1b is a leaderless protein thus it is not possible to exit the cell by using the classical Golgi route. IL-1b requires a proteolytic cleavage by the intracellular protease, caspase-1[54].

In recent years, increasing evidence exist about the key role that $P2X_7$ plays in this process. The first observation was that Lipopolysaccharides (LPS) primed macrophages managed only to synthesize a 33kD precursor of IL-1b that remained in the cytosol[55]. The

addition of ATP resulted in a massive release of the biologically active 17kD form of IL-1b to the pericellular space by macrophages and other cells[56-60]. Further experiments revealed that it was specifically $P2X_7$-receptor that mediated this ATP-driven mature IL-1b release[61-64]. Overexpression of $P2X_7$ receptors results in a massive release of mature IL-1b through secretory lysosomes within minutes and its absence prevents the secretion of IL-1b[65].

In summary the following are the major steps, currently known of the coplex IL-1b maturation and release pathway shown in Figure 1:

1. Toll-like receptors, like TLR4, become activated by pathogen associated molecular patterns (PAMPs) or lipopolysaccharide, bacterial endotoxin (LPS) and this activation leads to the production of pro-IL-1b and $P2X_7$ and panx1 activation[66-68].

2. When extracellular concentrations of ATP reach the micromolar range required for $P2X_7$ activation, a non selective cation channel opens and Ca++ and Na+ influx as well as K+ efflux begins thereby decreasing intracellular K+ levels.

3. Panx1 is activated and allows a dye uptake pathway and bacterial PAMPs and extracellular ATP enter the cell and activate the nucleotide leukin rich polypeptide 3 (NLRP3) inflammasome - a reaction facilitated by the low intracellular K+ levels- (nucleotide binding domain and leucine rich repeat containing a pyrin domain) using the apoptosis associated speck like protein (ASC) containing caspase recruiting domain (CARD)[69-72].

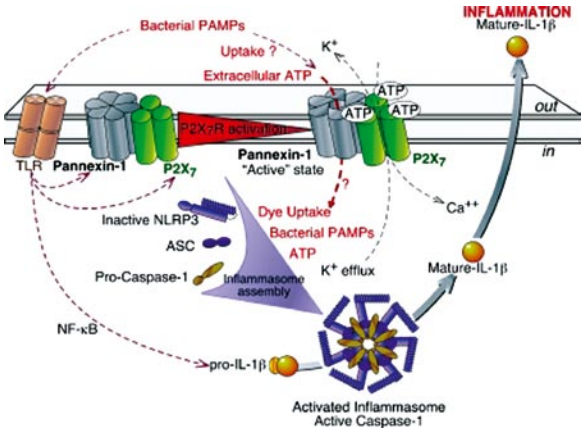

Figure 1: Pelegrin and Suprenant, Purinergic Signalling 2009 (with permission).

Once the inflammasome is assembled and activated, caspase1 is produced and enzymatically converts pro-IL-1b to its mature form that finally exits the plasma membrane either via exocytosis or via microvesicles. Several theories propose that IL-1 is released by apoptotic cell death and shedding of microvesicles[73, 74]. Exocytosis of secretory lysosomes[75-77] where proIL-1b is transported to endosomic vesicles together with caspase 1. In this protected compartment it is proteolytically cleaved and converted to the mature form.

The mechanism whereby this receptor is activated under physiological conditions is currently unknown[78]. The theory presumes the receptor acts as a danger sensor and ATP as a danger signal, as rather high ATP concentrations are required to activate $P2X_7$-receptor. This may create a vicious cycle where the receptor decides to perpetuate or halt the inflammatory process.

$P2X_7$-receptor and disease

There is an increasing body of evidence implicating $P2X_7$ receptor in various pathological conditions, such as: rheumatoid arthritis and chronic obstructive pulmonary disease, where inflammation is the cornerstone of these disorders. Selective $P2X_7$ antagonists are currently being investigated in clinical trials due to its close connection to IL-1b and TNF-a production[79, 80].

In neurological disorders, studies in rodent models revealed a close connection of $P2X_7$ to Alzheimer disease[81], Parkinson's disease[82], multiple sclerosis[86], sensory neuropathies[87], and neuropathic pain[83-85]. In cancer where apoptotic cell death is an important mechanism of disease, $P2X_7$ with its direct effect in apoptosis plays a significant role as it was shown in skin cancers[88,89] and uterine epithelial cancers compared to normal tissues. Perhaps $P2X_7$ will be of future use as abiomarker to distinct normal from cancer uterine epithelial tissues[90]. Early apoptotic cell death to the retina in diabetes in rodent models has been linked to $P2X_7$ activation in that part of the eye, suggesting apossible connection to diabetic microvascular injury[91].

Reported data for $P2X_7$ receptor and hypertension

1. A connection to aldosterone-mediated signaling to distal renal tubules was found;[92]

2. $P2X_7$ receptor polymorphisms may be linked to hypertension. A family based quantitative genetic association study involving 248 families was performed to investigate a possible association between $P2X_7$ genes (and P2X4, P2X6) and ambulatory blood pressure. Significant evidence of association between the single nucleotide polymorphism rs591874 in the first intron of the $P2X_7$ gene and blood pressure was found. The strongest association was found for nocturnal diastolic blood pressure although an association was present for both systolic and diastolic blood pressures measured by an observer during the day and at night[92].

3. Production of proinflammatory cytokines and promotion of apoptosis to endothelial cells may be linked to vascular remodeling in hypertension[93]. A possible link to hypotensive responses in inflammatory diseases via IL-1b release with nitric oxide synthesis may be postulated[44].

$P2X_7$ receptors are expressed in cells of the cardiovascular system and drugs affecting this signaling system may provide promising new therapies in hypertension and prevention of thrombotic events[94].

Current findings for $P2X_7$ associated with renal dysfuntion

The following findings are in favour of the purinergic signalling involvement in the pathogenesis of renal disease:

1. Under physiological conditions renal cells may release ATP that is found in tubular fluid and the final urine at a concentration that approximately reaches 200nM[95, 98] as well as several ectonucleotidases at cell surfaces and in tubular fluid[96,99].

2. When inflammation is present, several new events are taking place in the inflammatory milieu such as: ATP degrading enzymes are downregulated thus prolonging its action, and ionotropic $P2X_7$ needs only milliseconds to act as it does not require a second intracellular messenger[100]. Large amounts of ATP are released by dying or stressed cells[101,102], $P2X_7$ activation threshold decreases thus facilitating ATP binding at lower concentrations[103, 104]. Proinflammatory cytokines and bacterial products upregulate $P2X_7$ expression and increase its sensitivity to ATP[105,106].

3. Once activated, $P2X_7$ may become a self-activating source of ATP, as concentrations released at the cell surface of living cells reach 100–200 μM[108].

The combined published clinical data investigating the role of $P2X_7$ in disease demonstrate that in pathological conditions, $P2X_7$ activation may happen more frequently than predicted by studies derived from in vitro experiments.

Polycystic kidney disease and $P2X_7$

1. Investigating nephrogenesis and renal cyst growth in a congenital mouse model of polycystic kidney disease, $P2X_7$ was detected in metanephric mesenchyme, in the collecting ducts in the later stages of nephrogenesis and receptor's expression was obvious between cysts suggesting a non apoptotic role of this receptor in cyst enlargement[107].

2. In a mouse model of autosomal recessive polycystic kidney disease (ARPKD) Hilman et al, activating and blocking $P2X_7$ receptors demonstrated that the activation of this receptor reduced the number of the cysts[96]. $P2X_7$ receptor's protein was upregulated in human fetal ARPKD model versus the normal fetal collecting duct.

3. Turner et al, investigated cyst lining cells from Han:SPRD (cy/+) rat model of polycystic kidney disease cyst lining cells, homozygotic and heterozygote rat kidneys. $P2X_7$ receptor's mRNA was found to be increased in heterozygote (cy/+) but not in homozygote(cy/cy) rat kidneys. $P2X_7$ receptors were clearly expressed in the above mentioned cyst lining cells of this model of renal cystic disease, and its expression was increased in the cystic tissue[108].

Further investigation will determine whether the $P2X_7$ receptors plays a role in cell turnover and tissue remodeling of the cysts[108].

Renal fibrosis and $P2X_7$

$P2X_7$ receptors have been linked to inflammation by its association to the synthesis of IL-1b and release in activated macrophages and in rat brain astrocytes. $P2X_7$ - receptors mRNA was detected in the kidney raising the possibility that it may be involved with TGF-β production[109], a major cytokine involved in renal fibrogenesis. Solini clearly pointed out a role for $P2X_7$ receptor activation on macrophage function and matrix formation in the models of glomerular disease used, and its association to TGF-β release[110].

The molecular basis of renal fibrosis is yet to be clarified so in 2006, Goncalves et al.[111] evaluated the role of this intriguing receptor to renal inflammation and fibrosis. They used a well established model of fibrosis the unilateral ureteral obstruction (UUO) that has been proven to produce a typical fibrotic picture, with infiltration of macrophages, increased extracellular matrix protein deposition and tubular atrophy[112-115].

They used C57BI6 mice as a wild type, $P2X_7$ (-/-) knockout mice and control mice. Goncalves, et al., found that in the animals lacking $P2X_7$ receptor, tubulointerstitial injury following UUO, significantly attenuated injury compared to WT animals, as well as myofibroblasts. Collagen deposition and TGF-β expression were significantly reduced. The population of myofibroblasts was significantly reduced in the knockout mice, implying that $P2X_7$ receptor presence is somehow involved in this epithelial to mesenchymal transition. This may involve IL-1b secretion,that has been found to promote fibroblast proliferation and collagen production[116,117].

$P2X_7$ expression in healthy and diseased kidney

Turner et al, published a paper in 2003 where they used polyclonal antibodies and immunohistochemistry to prove that there is little if any expression of $P2X_7$ receptors in healthy kidneys. Indeed a very low level of $P2X_7$ receptor immunoreactivity was detectable in a few glomeruli and this was the first study in native renal tissues rather than using cell cultures.

Later experiments provided evidence for $P2X_7$ receptor's expression in cultured mesangial cells in rodent models and showed that it can mediate ATP-induced cell death by apoptosis[118,119]. In cell cultures it has also been detected in mouse podocytes and medullary collecting duct cells.

Turner et al[120,121] showed the distribution of $P2X_7$ receptor in diseased renal tissue. They carried out their work in diabetic animals-rats with Streptozocin induced diabetes and hypertensive rats of transgenic (mRen2) models. This study proved expression of $P2X_7$ receptors in the glomeruli of the two rodent models of disease and electron microscopy showed that the predominant expression was in podocytes, endothelial and mesangial cells. Altered $P2X_7$ receptor expression and increased sensitivity to ATP-induced apoptosis have been reported in cultured fibroblasts exposed to high concentrations of extracellular glucose[111].

Solini et al., also investigated the role of $P2X_7$ receptor in fibroblasts derived from skin biopsies of diabetic patients[122]. They found that these fibroblasts had increased expression of P2X7 receptor, in addition to increased apoptosis, IL-6 secretion, shape changes and enhanced fibronectin, responses known to be mediated by the receptors. These finding unravel an interesting mechanism that alters the cellular and extracellular structural components of the arterial wall, and at the same time, generates a proinflammatory milieu.

Apoptosis is an important mechanism in the kidney, leading either to healing or to renal scarring, therefore regulation of this process could be important in normal tissue repair and remodeling after injury. The finding that $P2X_7$ expression was increased in the glomeruli of the TGR2 hypertensive rodent model and also in the diabetic animals, compared to control animals, may indicate a role for the $P2X_7$ receptor in glomerular repair by deleting damaged cells whilst simultaneously encouraging proliferation and repair.

Another study by Solini et al[110] demonstrated in mesangial cell cultures that BzATP agonist of $P2X_7$ receptors, both in normal and high glucose environment that extracellular matrix protein production and TGF-β production were increased, while the application of oxidized ATP had the opposite effect. This suggests a potential relationship between $P2X_7$ receptor and the major profibrotic cytokine TGF-β. Harada et al used BzATP in cell cultures of mesangial cells where typical ladders of oligonucleosomal fragments of extracted DNA were yielded confirming the assay that $P2X_7$ receptor activation in them induces apoptotic death in mesangial cells[118].

$P2X_7$ and experimental nephritis

In a mouse model of experimental nephritis, increased expression of $P2X_7$ receptor protein levels were detected as well as increased apoprtosis in glomeruli. In the same study, increased protein levels of the receptor were detected in both tubular and glomerular cells derived from renal biopsies from patients with autoimmune related glomerulonephritis[124].

Furthermore, in a rat model of proliferative glomerulonephritis increased mRNA levels of $P2X_7$ receptor were found to coincide with the onset of proteinuria and maximally increase mRNA levels of IL-1b on the 4th day after the injection of the nephrotoxic serum[124]. These findings suggest that $P2X_7$ receptor could be an important factor in the pathogenesis of glomerulonephritis, either by the creation of apoptosis or by regulating the proinflammatory cytokines production (Figure 2).

More recently a novel selective antagonist (A438079) as well as the knockout $P2X_7$(-/-) mice were used in an attempt to investigate the role of this receptor in a rodent model of experimental nephritis created with the injection of nephrotoxic serum[125]. Renal function, urinary MCP-1 levels, macrophage infiltration and a dramatic increase in proteinuria were all observed in the mice lacking the $P2X_7$ receptors comparing to wild type animals. The use of the antagonist prevented the antibody mediated glomerulonephritis in rats suggesting that in autoimmune renal injury perhaps the missing link to successful treatment involves $P2X_7$ receptors[125].

Conclusion

$P2X_7$ receptor could be viewed as a danger sensor, a key point, where the decision whether inflammation will proceed further or not is taken.

Perhaps a new era is emerging where novel anti-analgesic and anti-inflammatory therapies would be developed specifically targeting at $P2X_7$–receptors.

The field of $P2X_7$- receptor might be so broad that it includes neurological disorders, such as depression and Alzheimer disease, lung disease, such as copd, rheumatoid arthritis, diabetic microvascular damage, including the area of diabetic nephropathy, renal fibrosis and experimental nephritis, and cardiovascular disease.

Many questions are yet to be answered for this in-

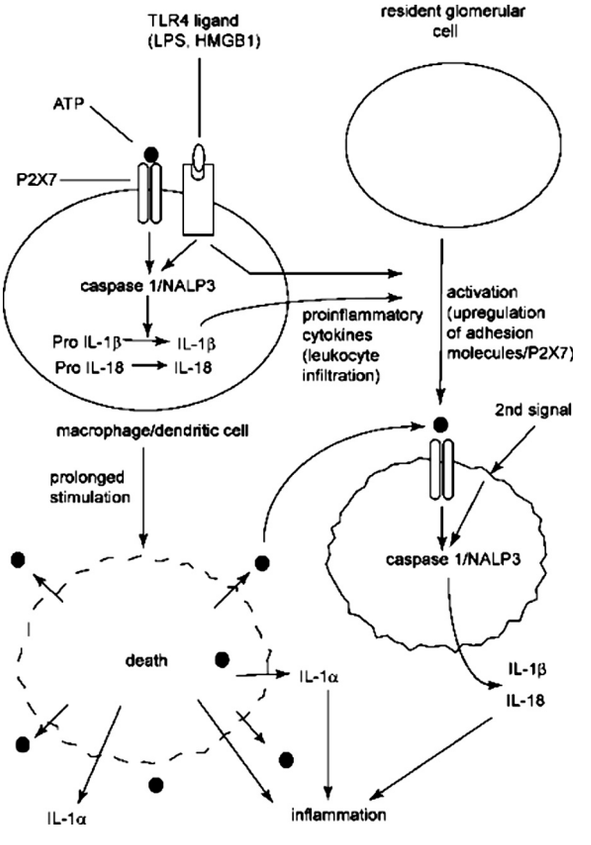

Figure 2: A possible mechanism of perpetuating renal inflammation via the activation of $P2X_7$.

ATP binding $P2X_7$ receptors in activated macrophages or dendritic cells activate $P2X_7$. This may lead to:

a) cell death in prolonged activation of $P2X_7$ and exacerbation of renal inflammation or b) to the activation of resident glomerular cells and the infiltration of leukocytes by proinflammatory cytokines released under the effect of activated $P2X_7$. c) $P2X_7$ is further upregulated, leading to caspase 1 activation expression and NLRP3 assembly releasing 1L-1b. d) 1L-1b exacerbates renal inflammation.

triguing receptor i.e. regarding the pore forming events and mutogenesis, mass spectroscopy, in vivo experiments with the novel selective antagonists and the knock in and knockout $P2X_7$ mice, will further contribute to unraveling the multiple aspects of $P2X_7$ receptor's involvement in disease.

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