DRUG DEVELOPMENT RESEARCH 53:72–76 (2001)

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Research Article

Genetic Polymorphisms of the Human $P2X_7$ Receptor and Relationship to Function

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Extracellular ATP has been shown to induce apoptotic death of many cell types of hematopoietic origin. This action of ATP is mediated via activation of $P2X_7$ purinergic receptors, which show the unusual property of time-dependent channel dilation to accept permeants as large as ethidium cation (314 Da). $P2X_7$ function, measured by area under the ATP-induced ethidium uptake curve, was 5-fold greater for monocytes than lymphocytes, while polymorphs and platelets showed no ethidium uptake. Expression of $P2X_7$ receptor, measured by the binding of a monoclonal antibody, was also 5-fold greater on monocytes than lymphocytes. However, in some subjects, both normal and with chronic lymphocytic leukemia, the $P2X_7$ receptor was nonfunctional despite good expression of $P2X_7$ protein. Three single nucleotide polymorphisms were found in the $P2X_7$ cDNA coding region, one of which correlated with $P2X_7$ function. Thus, the homozygous substitution of alanine for glutamic acid at amino acid 496 led to complete loss of function of the $P2X_7$ receptor, while the heterozygous polymorphism gave function half that of the germline $P2X_7$ receptor. Drug Dev. Res. 53:72–76, 2001.

Key words: $P2X_7$ receptor; non-functional $P2X_7$; polymorphisms of $P2X_7$; loss-of-function mutation

INTRODUCTION

Extracellular ATP mediates many biological responses ranging from smooth muscle contraction to synaptic transmission in the autonomic and central nervous systems [Burnstock, 1996; Dubyak and el-Moatassim, 1993]. The biological effects of ATP are becoming better defined in hematopoietic cells, where extracellular ATP has been shown to induce cytotoxicity in thymocytes [Nagy et al., 2000; Zheng et al., 1991], dendritic cells [Coutinho-Silva et al., 1999; Nihei et al., 2000], B-lymphocytes [Peng et al., 1998], and macrophages [Humphreys et al., 2000]. Many features of this ATP-mediated cytotoxicity resemble that produced by exposure of these cells to dexamethasone, namely, distinct morphological changes and fragmentation of target cell DNA usually associated with apoptosis [Wyllie, 1980]. These actions of ATP are mediated by the interactions of this nucleotide with $P2X_7$ purinergic receptors. The P2X receptor family have two transmembrane domains with intracellular amino and carboxyl termini and an oligomeric structure in the plasma membrane based on trimeric or larger complexes of identical subunits [Nicke et al., 1998]. The genes for the rat, mouse, and human $P2X_7$ receptors have now been cloned and show extensive homology (30–40%) with the other members of the P2X receptor family, although $P2X_7$ differs in having a long carboxyl terminus of 240 amino acids from the inner membrane face [Rassendren et al., 1997]. The genomic structure of $P2X_7$ consists of 13 exons with exon 12 and exon 13 coding for the carboxyl terminal tail of this molecule. There is strong evidence that this long carboxyl terminus is necessary for the permeability properties of the $P2X_7$ receptor since truncation of this tail abolishes ATP-induced uptake of

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the fluorescent dye Yo-Pro-1 [Surprenant et al., 1996]. The characteristics of the $P2X_7$ of monocyte-macrophages or of lymphocytes as well as HEK-293 cells expressing the cDNA for $P2X_7$ have shown features which are most unusual for a channel. These include the slow further dilatation following channel opening [Virginio et al., 1999; Wiley et al., 1998] and the activation of various proteases, including membrane metalloproteases [Gu et al., 1998] and intracellular caspases [Ferrari et al., 1999; Humphreys et al., 2000]. The fully dilated state of the $P2X_7$ pore accepts ethidium cation (314 Da) as a permeant, although the larger propidium^{2+} dye (414 Da) does not enter the channel during short-term incubations up to 60 min. The slow further increase in permeability that develops after an initial opening of the $P2X_7$ receptor channel has been studied by flow cytometric technique (Fig. 1), which offers unique advantages for studying permeability increase of the $P2X_7$ ion channel [Wiley et al., 1998]. Time-resolved flow cytometry generates the mean fluorescence intensity of 5,000 cells per 5-sec interval and this technique allows a sensitive measurement of the initial rates of ethidium uptake, which are essentially unidirectional because of binding of permeant cation to nucleic acids. An additional advantage of flow cytometry is that monocyte and lymphocyte subsets can be identified within the one population using two-color analysis with appropriate fluorescein-conjugated monoclonals (green 525 nm channel) used together with ethidium, whose uptake is measured on the red 570 nm channel. Figure 1 shows that the rapidly activated $P2X_7$ channel is initially impermeable to ethidium cation, but within the first 1 min after addition of ATP a steady increase in ethidium influx was observed. This delay in the rate of ethidium cation uptake contrasts with the immediate (<1 sec) fluxes of inorganic cations observed following ATP addition [Nuttle and Dubyak, 1994; Tatham and Lindau, 1990]. The area under the ATP-induced ethidium uptake curve is often used as a convenient measure of $P2X_7$ receptor function.

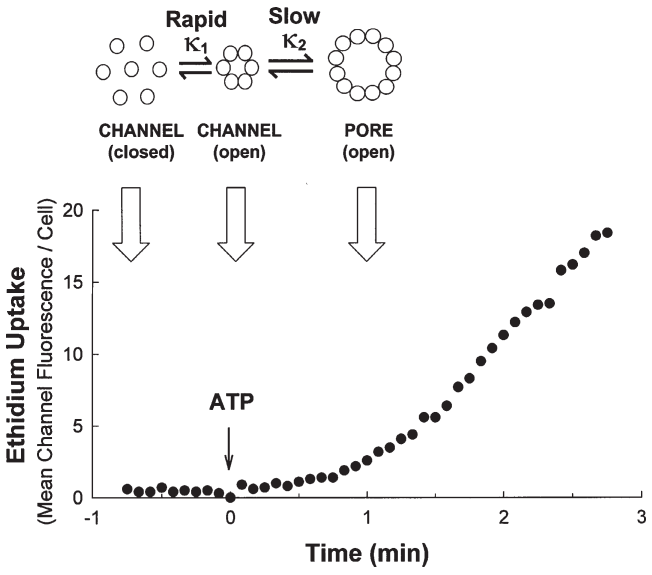

Fig. 1. A model for the $P2X_7$ receptor in which agonists activate immediate opening of a cation-selective channel, followed by a slower transition to a permeability pathway (pore) that passes larger cations up to the size of ethidium$^+$ and YO-PRO-1^{2+} cations. The rate of formation of the larger "pore" is slow ($k_2 << k_1$), so that the model predicts the second step (or series of steps) occurs at a rate ($k_2 \times P2X_{7open}$) that is largely determined by the abundance of the ligated (open) channels in the membrane. Partial agonists such as ATP maintain a lower proportion of the channels in the open conformation and thus show a time delay before inducing ethidium uptake. The ethidium uptake is measured as the mean channel of cell-associated fluorescence intensity at each 5-sec interval.

$P2X_7$ EXPRESSION AND FUNCTION IN LEUCOCYTES

When ATP-induced uptake of ethidium was measured into mononuclear cells of peripheral blood, a 5-fold greater uptake was seen into monocytes than into lymphocytes (Fig. 2) while there were no significant differences between the three major lymphocyte subsets (T-, B-, or NK- lymphocytes). ATP-induced uptake of ethidium into resting neutrophils or platelets was negligible. Addition of a monoclonal antibody known to inhibit the function of the $P2X_7$ receptor, reduced ATP-induced ethidium uptake almost to the basal level, confirming that ethidium permeates via the $P2X_7$ pathway. The surface expression of $P2X_7$ was measured by flow cytometry of normal white cells stained with directly conjugated $P2X_7$ monoclonal antibodies. Monocytes showed the highest expression of surface $P2X_7$ receptors, which was 4–5-fold higher than $P2X_7$ values for normal lymphocytes [Gu et al., 2000]. Polymorphonuclear neutrophils showed weak expression for $P2X_7$ when gated to exclude those cells

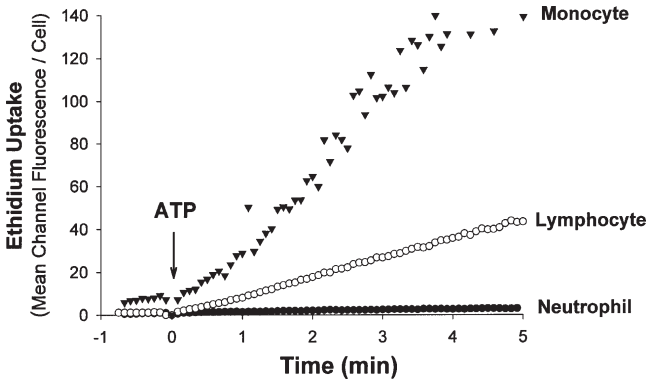

Fig. 2. ATP-induced ethidium uptake by monocytes, lymphocytes, and polymorphonuclear neutrophils from a normal subject. Mononuclear preparations (2×10^6/mL) prelabeled with cell type specific antibodies were suspended in HEPES-buffered isotonic KCl medium at 37°C. Ethidium was added followed 40 sec later by 1.0 mM ATP. Mean channel of cell-associated fluorescence intensity was measured for each gated population at 5-sec intervals.

that expressed CD41 as a result of platelet adhesion. Large amounts of $P2X_7$ protein were found in an intracellular location in monocytes and lymphocytes of all subtypes and the intracellular expression was approximately an order of magnitude greater than expression on the cell surface. When expression of $P2X_7$ receptors on the surface of normal leukocyte subsets was compared with the ATP-induced uptake of ethidium, a close correlation was found [Gu et al., 2000].

NONFUNCTIONAL $P2X_7$ IN SOME SUBJECTS

We also studied the $P2X_7$ expression and function on B-lymphocytes from patients with chronic lymphocytic leukemia (CLL), a clonal disease in which mature B-lymphocytes accumulate in blood and bone marrow. In most patients, the leukemic cells express surface $P2X_7$ receptors at about the same level as observed for normal B-lymphocytes. Generally, these $P2X_7$ receptors on leukemic B-lymphocytes were fully functional as judged by ATP-induced ethidium uptake. However B-lymphocytes from three patients failed to take up ethidium in the presence of ATP. This absence of $P2X_7$ function was not due to lack of surface $P2X_7$ expression since the binding of fluorescein-conjugated $P2X_7$ monoclonal antibodies was no different to that found for normal B-lymphocytes. One possible explanation was that a circulating inhibitory factor, possibly a cytokine, may have inhibited the function of the $P2X_7$ receptor in these three patients. Incubation for 24 h of lymphocytes from patients with functional $P2X_7$ in buffered medium containing 80% plasma from patients with nonfunctional $P2X_7$ failed to inhibit the $P2X_7$ response (Fig. 3). Buell et al. [1996] showed that ambient ATP released during incubation of HL-60 cells produced a sustained desensitization of $P2X_1$ receptors. However, addition of apyrase (5 U/ml) during incubation of B-CLL lymphocytes failed to reactivate the functionally inactive $P2X_7$ in these cells.

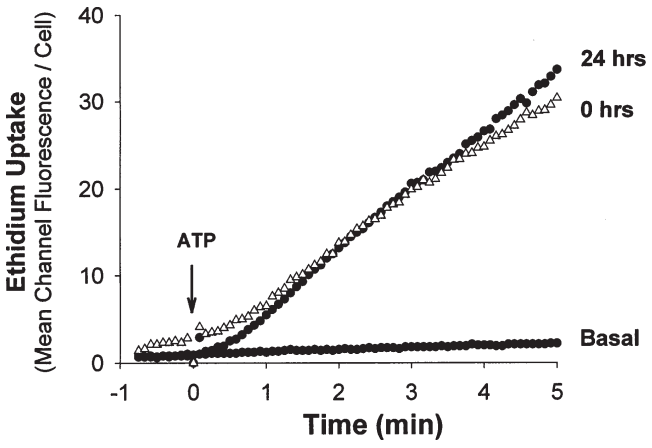

Fig. 3. ATP-induced ethidium uptake by B-lymphocytes from a $P2X_7$ functional subject incubated with 80% plasma from a $P2X_7$ nonfunctional subject for 0 and 24 h. Ethidium was added followed 40 sec later by 1.0 mM ATP. Mean channel of cell-associated fluorescence intensity was measured for the gated $CD19^+$ lymphocyte population at 5-sec intervals.

LOSS-OF-FUNCTION POLYMORPHISM

An alternative possibility is that genetic changes may underlie the loss of function of the $P2X_7$ receptor in certain subjects. For this reason the sequence of cDNA for the coding region of the $P2X_7$ gene was analyzed both in the three CLL patients and in a large cohort of normal subjects. Three single nucleotide substitutions were found in several of the normal and CLL subjects at position corresponding to amino acids 155, 348, and 496 (Fig. 4). One of these substitutions (C489T) has previously been described by Buell et al. [1998]. All three substitutions occurred at allele frequencies greater than 0.01 (1%) in the population and could thus be defined as single nucleotide polymorphisms (Table 1). Single nucleotide polymorphisms are increasingly recognized as a source of genetic variation and their density may be as high as one per kb of cDNA [Collins et al., 1999]. Most of these polymorphisms have a neutral effect on function, but some contribute to loss of protein function.

Because the long carboxyl-terminal tail of the $P2X_7$ receptor regulates its permeability properties [Surprenant et al., 1996], the polymorphism at amino acid 496 (Glu496Ala) was of particular interest. Thus, a PCR product was amplified directly from DNA between nucleotide 1425 and 1780 of the coding region of the $P2X_7$ gene and the product was sequenced. In 9 of 45 subjects a heterozygous nucleotide substitution (adenine to cytosine)

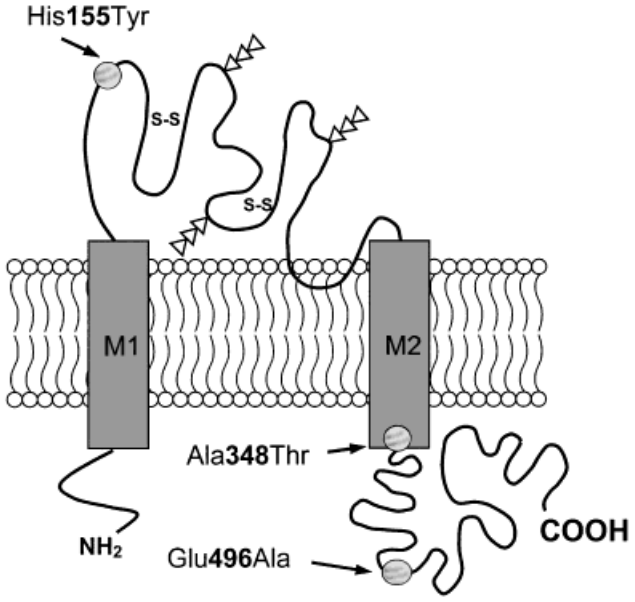

Fig. 4. Three single nucleotide polymorphisms found in $P2X_7$ receptor: His155Tyr in the extracellular domain, Ala348Thr in the end of second transmembrane domain, and Glu496Ala in the long C-terminal tail.

TABLE 1. Allele Frequencies of $P2X_7$ Polymorphic Sites

Normal subjects	Nucleotide polymorphism	Amino acid	Allele frequency
n = 9	C489T	Histidine (155)	0.44
		Tyrosine (155)	0.56
n = 5	G1068A	Alanine (348)	0.4
		Threonine (348)	0.6
n = 45	A1513C	Glutamic acid (496)	0.88
		Alanine (496)	0.12

was found at position 1513, while in 1 out of 45 subjects a homozygous A1513C substitution was observed (Table 1). Homozygosity for the mutation led to almost complete loss of function of the receptor, while heterozygosity for the mutation gave a function approximately half that of the germline $P2X_7$ sequence (Fig. 5). Moreover, one of the three patients with B-CLL and absent $P2X_7$ function carried the A1513C mutation in homozygous dosage, confirming the strong association of this polymorphism with the function of the $P2X_7$ receptor.

CONCLUSIONS

Loss-of-function mutations can affect genes encoding ion channel proteins [Lester and Karschin, 2000]. For example, three loss-of-function mutations have been identified in the human $K_{1R}6.2$ gene which encodes the two-transmembrane protein subunit of the pancreatic β-cell ATP sensitive K^+ channel [Sharma et al., 2000]. In this study, a single nucleotide polymorphism changing glutamic acid to alanine at amino acid 496 of the human $P2X_7$ receptor affected its function. Homozygosity (C/C) for this polymorphic mutation led to almost complete loss of $P2X_7$ function in leukocytes, while heterozygosity (A/C) gave a function which was half that of cells with the germline $P2X_7$ sequence. There is good evidence that activation of macrophage $P2X_7$ receptors by ATP can produce killing of intracellular *Mycobacteria tuberculosis* by these cells [Lammas et al., 1997], and it is possible that the polymorphism described above may turn out to be a susceptibility factor predisposing individuals to *Mycobacterial* infections.

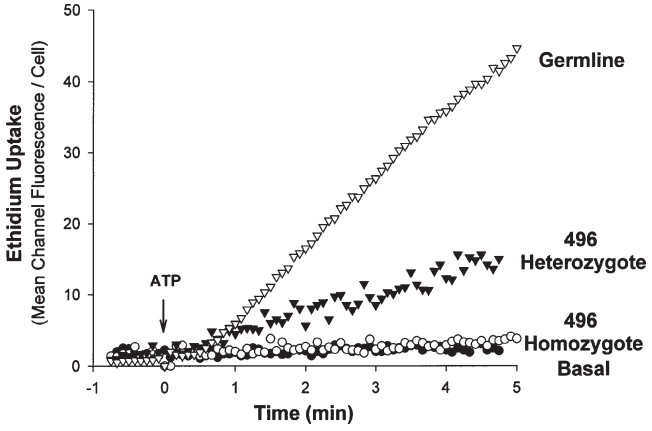

Fig. 5. Typical ATP-induced ethidium uptake curve in B-lymphocytes from normal subjects with germline, Glu496Ala heterozygous, and Glu496Ala homozygous $P2X_7$ as indicated. Ethidium was added followed 40 sec later by 1.0 mM ATP. Mean channel of cell-associated fluorescence intensity was measured for the gated $CD19^+$ lymphocyte population at 5-sec intervals.

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