Specific detection of non-functional human $P2X_7$ receptors in HEK293 cells and B-lymphocytes

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Received 9 January 2003; revised 12 February 2003; accepted 12 February 2003

First published online 24 February 2003

Edited by Veli-Pekka Lehto

Abstract **$P2X_7$ receptor/channels mediate ATP-induced apoptosis in a range of cells including lymphocytes. HEK293 cells were transfected with wild-type human $P2X_7$ receptor or site-directed mutant constructs (K193A, K311A and E496A) known to be non-functional from measurements of barium/ethidium influx in the presence of ATP or 2′,3′-*O*-(4-benzoylbenzoyl)-ATP. An antibody was designed against an epitope from a loop adjacent to the extracellular ATP site. The epitope was unavailable in cells expressing normal functional surface receptors. Non-functional surface receptors as well as intracellular receptors selectively bound the antibody. So did B-lymphocytes from chronic lymphocytic leukemia patients expressing non-functional (E496A) mutant receptor.**

Key words: $P2X_7$; Channel function; Cytolytic pore; Antibody

1. Introduction

Purinergic receptors of type P2X are fast, ligand-gated cation channels that open in response to the binding of extracellular ATP. The seven subtypes designated $P2X_{1-7}$ exhibit extensive homology (30–40%), particularly in structurally important elements such as disulfide bridges in the extracellular domain located between the two transmembrane domains [1]. Each subtype has a short N-terminal sequence but the long intracellular C-terminal domain is unique to $P2X_7$ and is thought to confer time-dependent dilation of the channel into a pore that is able to transport large organic cations such as ethidium and Yo-Pro (quinolinium, 4-[(3-methyl-2(3*H*)-benzoxazolylidene) methyl]-1-[3-(trimethylammonio) propyl]-diiodide) with molecular weights of up to 375 Da [2,3]. $P2X_7$ receptors form trimeric or larger homomeric structures [4] that are found in a range of cells including astrocytes [5], endothelial cells [6], rat prostatic epithelial cells [7], monocytes [8], lymphocytes [9] and macrophages [10]. A mutation at position 496 in the carboxy-terminal tail leads to loss of the permeability properties of $P2X_7$ for both small and large inorganic cations [11]. Similarly, site-directed mutagenesis of either of the two lysines K193 and K311 fully inhibits the normal function of $P2X_7$ by affecting the binding of agonist through the phosphate side chain of ATP or ATP analogues [3]. Moreover, residue 214 was found to be associated with an ATP binding site based on marked species differences in agonist dissociation rates between rat, mouse and human $P2X_7$ [12].

While the exact location of the ATP binding pocket in $P2X_7$ is uncertain, the segment around 193–214 was examined for suitability as an epitope that may alter its exposure to an antibody in the presence of bound agonist in order to discriminate between surface receptors that are active and those that are inactive in the absence of bound agonist. The successful production of an antibody that binds only to non-functional receptors as measured by their ability to form pores capable of taking up barium/ethidium provides structural insight into the mechanism of action of agonist and shows that cells expressing non-functional cytolytic $P2X_7$ may be detected selectively using this probe.

2. Materials and methods

2.1. Materials

ATP, BzATP, ethidium bromide, barium chloride, RPMI 1640 medium, D-glucose, and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA); Ficoll-Hypaque (density 1.077), GFX™ PCR DNA and Gel Band Purification Kit from Amersham Pharmacia (Uppsala, Sweden); Cy3-conjugated anti-rabbit Ig antibody from Jackson ImmunoResearch (West Grove, PA, USA); Affigel 10 from Bio-Rad (Hercules, CA, USA); HEPES, LipoFectamine™ 2000 reagent, Taq DNA polymerase, Opti-MEM I medium from Life Technologies (Gaithersburg, MD, USA); The Wizard Genomic DNA Purification Kit from Promega (Madison, WI, USA); QIAquick Gel Extraction Kit from Qiagen (Australia); QuikChange™ Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA).

2.2. Antibody production

Using identical methods as described earlier [5], an antibody was raised in rabbit to the epitope G200–C216 in human $P2X_7$. The presence of a proline at 210 confers a *cis*/*trans* isomerization on the peptide. Only the *cis* conformer produced an antibody that recognized the non-functional receptors since site-directed mutagenesis of Pro210 to lock the peptide in a *trans* configuration produced a P210A $P2X_7$ receptor that was fully functional [3] and that was unable to bind the antibody. Specificity of the antibody was checked in the usual ways [5] with adsorption with conjugate peptide and free peptide eliminating binding. Affinity purification of the protein G-purified IgG fraction was carried out using the epitope bound to Affigel-10 (Bio-Rad). Western blots of HEK cells transfected with mutant E496A or K193A $P2X_7$ receptor show a single band corresponding to the full-length protein at 80 kDa.

2.3. Tissue culture

HEK293 cells were transfected with cDNAs encoding human wild-type or a mutant construct of $P2X_7$ receptors using LipoFectamine

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0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00172-8

2000. Cells were maintained in RPMI 1640 complete medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 0.02 mg/ml gentamicin at 37°C, 5% CO_2. Truncated, germline and mutant pCI-P2X$_7$ plasmids were transfected into HEK293 cells as described [11].

2.4. Site-directed mutagenesis

The full-length clone of hP2X$_7$ (GenBank accession number Y09561) was prepared as described [11]. The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's instructions to perform point mutations in the hP2X$_7$ receptors at K193, P210, K311 and E496 as described [3]. Site-directed mutations were confirmed by sequence analysis.

2.5. Immunofluorescent staining

Immunofluorescent staining and confocal microscopy of HEK293 cells and human B-lymphocytes incubated on collagen-coated glass coverslips were performed as described [13,14] using a P2X$_7$ polyclonal antibody [14] and the new antibody to non-functional receptors. Cells were visualized with a Leica TCS NT UV laser confocal microscope system as previously described [13].

2.6. Ethidium influx measurement by flow cytometry

Flow cytometry was used to quantitate ethidium bromide uptake as described [15]. Briefly, cells (10^6) were labeled with the appropriate antibody and were then washed once and resuspended in 1 ml of buffer containing 10 mM HEPES, 150 mM KCl, 5 mM D-glucose, 0.1% bovine serum albumin, pH 7.5 and 37°C. Cells were analyzed at 1000 events/s on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). They were gated by forward and side scatter. All samples were stirred. Ethidium (25 μM) was added followed after 40 s by 1 mM ATP. Linear mean channel of fluorescence intensity for each gated subpopulation over successive intervals of 5 s was analyzed using WinMDI software (Joseph Trotter, V 2.7) and plotted against time.

2.7. Barium influx using Fura-red

HEK293 cells and B-lymphocytes (4×10^6) were incubated with Fura-red (1 μg/ml) for 30 min at 37°C in HEPES-buffered NaCl medium as described previously [11].

2.8. Patient samples

Heparinized venous blood from chronic lymphocytic leukemia (CLL) patients and normal volunteers was diluted with an equal volume of RPMI 1640 medium and the mononuclear cells separated by density gradient centrifugation as described [14].

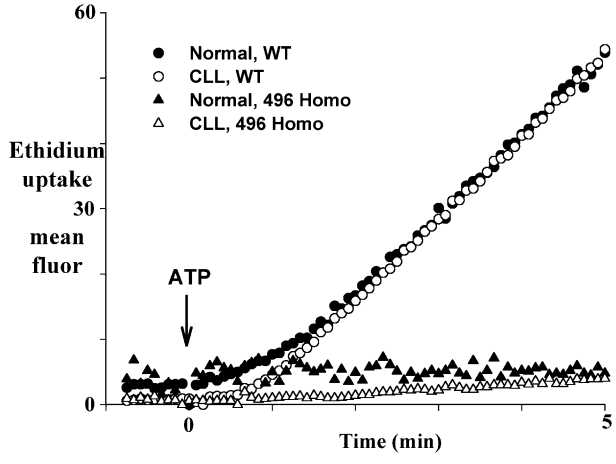


Fig. 1. ATP-induced ethidium uptake into B-cell lymphocytes. Mean channel fluorescence intensity (ordinate) measured over 5 s intervals for normal wild-type P2X$_7$ (closed circles), normal CLL P2X$_7$ (open circles), normal, E496A homozygous P2X$_7$ (filled triangles) and CLL E496A homozygous P2X$_7$ (open triangles).

3. Results

3.1. Functional analysis of pore formation of P2X$_7$ on B-cells by ATP-induced uptake of ethidium

Uptake of the fluorescent dye ethidium$^+$ measured by flow cytometry was used as an indicator of the flux of a larger permeant through the P2X$_7$ pore. ATP did not stimulate ethidium uptake in B-cells expressing homozygous mutant P2X$_7$ E496A from samples derived from blood taken from either normal subjects or CLL patients. In contrast, ATP produced a substantial increase in the rate of ethidium influx in B-cells taken from normal subjects and CLL patients expressing wild-type P2X$_7$ (Fig. 1). Identical results were obtained (data not shown) using fluorometry in which the entry of Ba^{2+} via the P2X$_7$ channel in cells loaded with Fura-2 was examined in both HEPES-buffered KCl buffer and sucrose buffer [16]. The 496 mutant receptors in normal and CLL patients were unable to take up Ba^{2+}. Identical results have been shown using HEK293 cells transfected with K193A and K311A mutant P2X$_7$ while the P201A mutant was found to be functional [3].

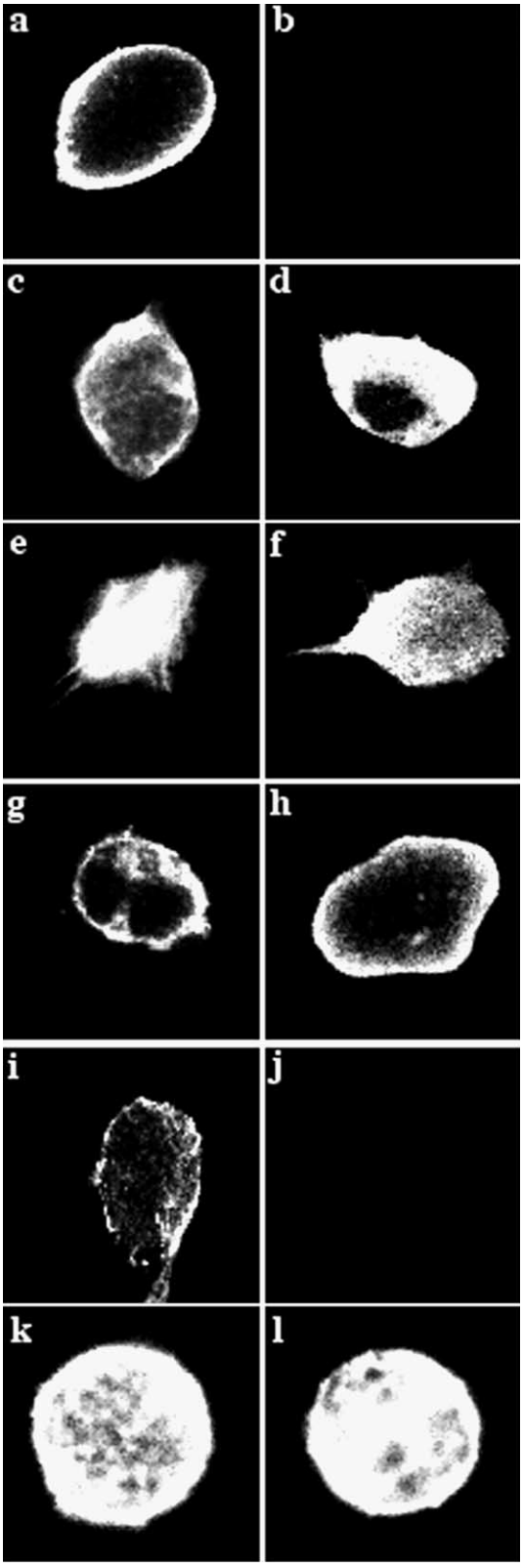
3.2. Expression of P2X$_7$ constructs in transiently transfected HEK293 cells

Confocal microscopy showed wild-type P2X$_7$ (Fig. 2a) and mutants E496A (Fig. 2c), K193A (Fig. 2e), K311A (Fig. 2g) and P210A (Fig. 2i) expressed equally strongly on the surface of HEK293 cells where they were labeled using Cy3 bound to a polyclonal P2X$_7$ antibody conjugated via anti-rabbit IgG. Negative controls included non-transfected cells and wild-type cells labeled in the presence of 10 μM adsorption control peptide epitope. Under these conditions all binding was abolished. The right-hand panels show cells labeled with the antibody to non-functional P2X$_7$. No binding occurs on the surface of wild-type cells (Fig. 2b). This negative staining is identical to the results obtained with non-transfected HEK cells either permeabilized or non-permeabilized labeled with either antibody and is also the same as the result found in staining the functional P210A receptors (Fig. 2j). The three non-functional receptors E496A (Fig. 2d), K193A (Fig. 2f) and K311A (Fig. 2h) reveal clear binding on the cell surfaces. Confocal microscopy of fixed and permeabilized HEK cells expressing wild-type P2X$_7$ showed that the general purpose polyclonal P2X$_7$ antibody (Fig. 2k) bound as well as the antibody to non-functional receptor (Fig. 2l) indicating that both epitopes are available on intracellular P2X$_7$ protein.

3.3. Surface expression of P2X$_7$ in B-lymphocytes

Confocal microscopy was employed to demonstrate that wild-type B-cells expressed P2X$_7$. The receptors were labeled on the cell surface using anti-rabbit Cy3-bound IgG secondary to a polyclonal P2X$_7$ antibody (Fig. 3a). Identical results were obtained in labeling wild-type CLL B-cells (Fig. 3c), B-cells from normal subjects who were homozygous for the E496A mutation (Fig. 3e) and B-cells from CLL patients who also were homozygous for the E496A mutation (Fig. 3g). The right-hand panels show cells labeled with the antibody to non-functional P2X$_7$. Both wild-type normal (Fig. 3b) and wild-type CLL (Fig. 3d) expressed P2X$_7$ receptors but the antibody selective for non-functional receptors was unable to bind to them. In contrast, the antibody selective for non-functional receptors was able to bind to normal (Fig. 3f) and CLL

(Fig. 3g) B-cells homozygous for the E496A mutation that have no pore function. The binding of each antibody to these non-functional receptors was equally strong.



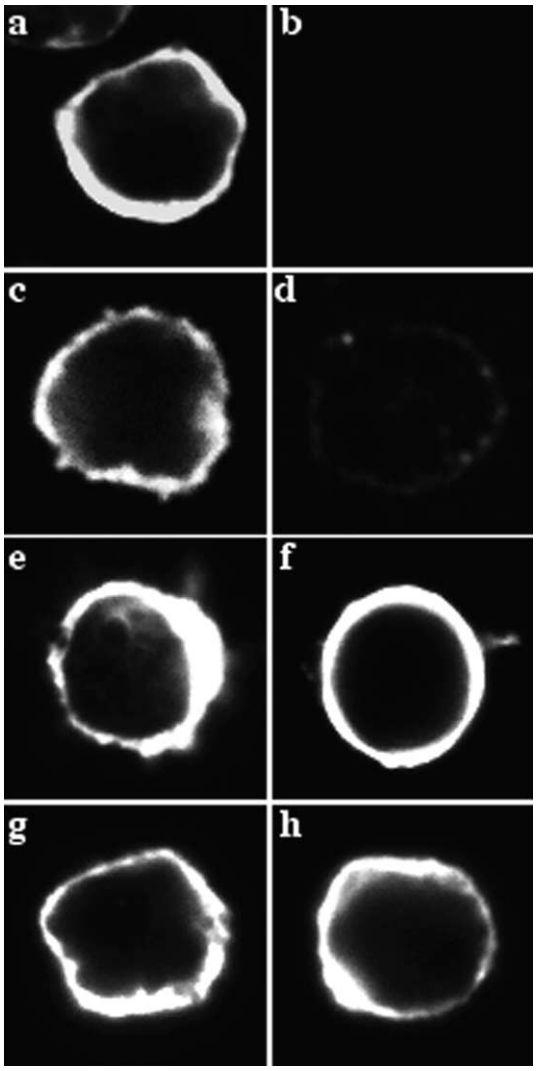


Fig. 3. Confocal microscope images of human and mutant $P2X_7$ receptor expression on the surface of B-cell lymphocytes. B-cell lymphocytes from normals expressing either (a,b) fully functional wild-type $P2X_7$ or (e,f) non-functional E496A mutant $P2X_7$, and B-cell lymphocytes from B-CLL patients expressing either (c,d) fully functional wild-type $P2X_7$ or (g,h) non-functional E496A mutant $P2X_7$ using either an antibody directed against a non-conformationally sensitive epitope on $P2X_7$ (left-hand panels) or an antibody designed to detect non-functional receptor (right-hand panels). All cells were labeled with the rabbit primary and subsequently with Cy3-conjugated anti-rabbit IgG antibody. Field widths are 10 μm.

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Fig. 2. Confocal microscope images of human and mutant $P2X_7$ receptor expression on HEK293 cells. Non-permeabilized HEK293 cells transiently transfected with either (a,b) wild-type, (c,d) E496A, (e,f) K193A, (g,h) K311A, (i,j) P210A using either an antibody directed against a conformationally insensitive epitope on $P2X_7$ (left-hand panels) or an antibody designed to detect non-functional receptor (right-hand panels). All cells were labeled with one of the rabbit primaries and subsequently with Cy3-conjugated anti-rabbit IgG antibody. Field widths are 25 μm. Permeabilized HEK293 cells transfected with wild-type $P2X_7$ were similarly labeled (k,l) showing the non-functional receptor antibody detects intracellular protein. Field widths are 10 μm.

4. Discussion

Surface expression of $P2X_7$ receptors on lymphocytes may be maintained at normal levels while still exhibiting a loss of function [11]. In normal leukocytes a close correlation is observed between ATP-induced ethidium uptake and surface expression of $P2X_7$ receptors [17]. The inability of the receptor/channels to form cytolytic pores may result from reduced availability of ATP [3] and/or a loss of control in the $P2X_7$-associated anchorage proteins [11]. The removal of a positive charge at the extracellular ATP binding site caused by substitution of alanine for lysine at either 193 or 311 or the removal of a negative charge at an intracellular location caused by the mutation of a glutamic acid (496) into an alanine appears equally efficient at abolishing the ATP-induced uptake of both small and large cation permeants, the former through direct effects on charge conservation at the ATP site and the latter due to presumed disruption to channel packing. The K193A and K311A mutant receptors are clearly non-functional, being entirely unable to form cytolytic pores while the E496A mutant receptors can re-establish some pore formation at very high expression levels in HEK cells, although not when expressed as homozygous mutant $P2X_7$ receptors in B-lymphocytes (unpublished results). The antibody specifically designed to bind to non-functional receptors readily complexes with intracellular (non-functional $P2X_7$) receptors indicating that the epitope is available on monomers or intracellular aggregates in the absence of bound ATP as well as on the above mutant receptors trafficked to and inserted in the plasma membrane. Thus there are no impediments to antibody binding by possible glycosylation sites on the epitope. Rather, the epitope is normally conformationally unavailable in wild-type functional expressed receptor.

Of the 20 amino acids, proline is the most likely to adopt the *cis* isomeric form because of its unique side chain that is linked to the main chain nitrogen thereby acting to reduce the energy difference between the *cis* form and the more stable *trans* form. *Cis*-proline has about 25% of the stability of *trans*-proline, very much higher than any of the other amino acids. *Cis–trans* isomerization is the rate-limiting step in the folding of some proteins. Many proteins such as Pin1/Cdc25 [18] and ribonuclease A at Pro 93 [19] utilize *cis–trans*-proline isomerization to regulate protein function. In the chosen $P2X_7$ epitope segment adjacent to the ATP binding site from 200 to 216, only one proline is present (Pro210). As discussed, this segment is exposed only in the absence of bound ATP being completely hidden in functional receptors expressed on the plasma membrane. Pro210 is conserved in mouse, rat and human $P2X_7$ sequences. Locking Pro210 in *trans* by exchanging proline for alanine by site-directed mutagenesis produces a functional receptor [3]. Moreover, the antibody designed specifically for non-functional receptors is unable to bind to this P210A mutant due to the non-exposure of the epitope in *trans* configuration. This indicates that there are no components to a *trans* conformer remaining in the purified serum. It therefore appears likely that Pro210 adopts a *trans* configuration in functional receptors where ATP is bound but a *cis* configuration in non-functional receptors where ATP is unbound. Furthermore, replacement of Ile214, adjacent to Pro210 in the selected epitope, affects dissociation of agonist binding [12] thereby affecting the potency of ATP, further indicating that Ile214, at the end of the epitope sequence, is associated with an ATP binding site.

Switching between functional and non-functional receptor conformations through *cis*/*trans* conformational change in Pro210 seems to apply irrespective of whether the lack of observed pore function is caused by lack of bound ATP or by disruption to $P2X_7$ monomer packing within the channel/pore. Thus inhibition of intracellular anchorage protein packing in homozygous E496A $P2X_7$ receptors in lymphocytes appears to interfere with the ability of ATP to bind to the extracellular domain. It may be that the monomers are unable to form appropriate intermonomer ATP sites leaving the epitope 200–216 exposed and thus able to bind the antibody to non-functional receptors. Other conformationally sensitive antibodies have been described, usually developed by appropriate selection of monoclonals [20,21], but we have shown that careful selection of structural epitopes can achieve the same result with a polyclonal antibody.

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