An Arg<sup>307</sup> to Gln Polymorphism within the ATP-binding Site Causes Loss of Function of the Human P2X<sub>7</sub> Receptor* 

Received for publication, December 19, 2003, and in revised form, April 8, 2004
Published, JBC Papers in Press, April 27, 2004, DOI 10.1074/jbc.M313902200

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The P2X<sub>7</sub> receptor is a ligand-gated channel that is highly expressed on mononuclear cells of the immune system and that mediates ATP-induced apoptosis. Wide variations in the function of the P2X<sub>7</sub> receptor have been observed, explained in part by loss-of-function polymorphisms that change Glu<sup>496</sup> to Ala (E496A) and Ile<sup>568</sup> to Asn (I568N). In this study, a third polymorphism, which substitutes an uncharged glutamine for the highly positively charged Arg<sup>307</sup> (R307Q), has been found in heterozygous dosage in 12 of 420 subjects studied. P2X<sub>7</sub> function was measured by ATP-induced fluxes of Rb<sup>+</sup>, Ba<sup>2+</sup>, and ethidium<sup>+</sup> into peripheral blood monocytes or various lymphocyte subsets and was either absent or markedly decreased. Transfection experiments showed that P2X<sub>7</sub> carrying the R307Q mutation lacked either channel or pore function despite robust protein synthesis and surface expression of the receptor. The monoclonal antibody (clone L4) that binds to the extracellular domain of wild type P2X<sub>7</sub> and blocks P2X<sub>7</sub> function failed to bind to the R307Q mutant receptor. Differentiation of monocytes to macrophages up-regulated P2X<sub>7</sub> function in cells heterozygous for the R307Q to a value 10–40% of that for wild type macrophages. However, macrophages from a subject who was double heterozygous for R307Q/I568N remained totally non-functional. These data identify a third loss-of-function polymorphism affecting the human P2X<sub>7</sub> receptor, and since the affected Arg<sup>307</sup> is homologous to those amino acids essential for ATP binding to P2X<sub>7</sub> and P2X<sub>2a</sub>, it is likely that this polymorphism abolishes the binding of ATP to the extracellular domain of P2X<sub>7</sub>.

In cells of the hemic and immune systems, extracellular ATP can induce cytolysis of lymphocytes (1), monocytes/macrophages (2), and dendritic cells (3). It is generally accepted that these cytolytic effects of ATP are mediated by the P2X<sub>7</sub> receptor, which is a ligand-gated cation channel activated by extracellular ATP and highly expressed on these cell types (4, 5). The P2X<sub>7</sub> ionic channel opened by extracellular ATP shows strong selectivity for the divalent cations Ca<sup>2+</sup> and Ba<sup>2+</sup> over monovalent cations (6, 7). After immediate (<1 s) channel opening, in the presence of agonist, a second permeability state develops that allows larger organic cations to pass, a process termed “pore” formation (8–10). This larger permeability state allows permeation by the ethidium<sup>+</sup> cation (314 Da) or YO-PRO-1<sup>−</sup> (375 Da) but excludes passage of propidium<sup>2+</sup> (414 Da) into lymphocytes (11) and monocyte-derived dendritic cells (12). Studies of P2X<sub>7</sub> of monocytes/macrophages as well as human embryonic kidney (HEK)-293 cells expressing the cDNA for P2X<sub>7</sub> have shown that this molecule forms part of a membrane complex (13) that activates the caspase signaling cascade (2, 14, 15) as well as intracellular phospholipase D (PLD) (16, 17) and various proteinases such as membrane metalloproteinases that shed surface-L-selectin and CD23 (18, 19).

P2X receptors have an oligomeric structure in the plasma membrane based on trimeric or larger complexes of identical subunits (20, 21). Moreover the values of Hill coefficients derived from the sigmoid ATP dose-response curves of the P2X<sub>7</sub>-PZ<sub>2</sub> receptor are consistent with multiple ATP-binding sites in each P2X<sub>7</sub> trimer (8, 22, 23). All seven members of the P2X receptor family have two transmembrane domains with intracellular amino and carboxyl termini. Little is known of the conformation of the extracellular domain containing the ATP-binding site(s). An analysis of the P2X subtype sequence homology has shown that the two transmembrane domains M1 and M2 are separated by an extracellular sequence containing intracellular amino and carboxyl termini. Little is known of the conformation of the extracellular domain containing the ATP-binding site(s). An analysis of the P2X subtype sequence homology has shown that the two transmembrane domains M1 and M2 are separated by an extracellular sequence containing a cysteine-rich region (residues 110–170) followed by a segment from Phe<sup>368</sup> to Val<sup>371</sup> that may form six antiparallel β-sheet homologous with members of the class II aminocyl-tRNA synthetases (24). The ATP-binding site is very likely to lie in this β-sheet region since two positively charged residues, Lys<sup>193</sup> and Lys<sup>311</sup> have been identified as being associated with the ATP-binding site (25).

Our previous studies have shown that genetic factors play a role in the functional phenotype of the P2X<sub>7</sub> receptor. In around 20% of the population, a Glu<sup>496</sup> to Ala polymorphism (1513A→C), which is located in an ankyrin repeat motif of the carboxyl terminus of P2X<sub>7</sub> receptor (26), leads to loss of function in homozygous individuals and ~50% reduction in heterozygous individuals (27). A second polymorphism, Ile<sup>568</sup> to

* This work was supported by the National Health and Medical Research Council, the Cure Cancer Australia Foundation, the Leukemia Foundation of Australia, and a Sesqui Fellowship from the University of Sydney (to B. J. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HEK, human embryonic kidney; CLL, chronic lymphocytic leukemia; FITC, fluorescein isothiocyanate; PE, phycoerythrin; mAb, monoclonal antibody; hP2X<sub>7</sub>, human P2X<sub>7</sub>; PBS, phosphate-buffered saline; PLD, phospholipase D; BuATP, 2′,3′-O-(4-benzoyl)benzoyl ATP; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

This paper is available on line at http://www.jbc.org
Asn (1729T→A), lies in a trafficking motif of the carboxyl terminus (28) and prevents normal trafficking and surface expression of this receptor (29). In this study, we report a third polymorphism within exon 9 of the human P2RX7 gene that changes Arg235 to an uncharged glutamine at the homologous position to Arg235 in P2X2, and Arg235 in P2X9, both of which are essential for the binding of ATP and activation of these receptors (30, 31). Cells carrying the Arg235→Gln polymorphism have reduced or absent P2X7 function due to the failure of ATP binding to the extracellular domain of P2X7.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, BzATP, ethidium bromide, barium chloride, digoxin, n-glucose, bovine serum albumin, RPMI 1640 medium, gentamicin, collagen (Type X), glycerol gelatin mounting medium, 7-aminoactinomycin D, 6-aminoacaproic acid, Bistir, ε-amin-o-n-caproic acid (6-aminoacaproic acid), and n-dodecyl β-d-maltoside/laurylmaltoside were purchased from Sigma. Interferon-γ was from Roche Diagnostics. HEPES, fetal calf serum, normal horse serum, LipofectAMINETM 2000 reagent, Opti-MEM I medium, Tag DNA polymerase, and pcDNA3 plasmid vector were from Invitrogen. Ficoll-PaqueTM PLUS and a GFX™ PCR DNA and gel band purification kit were from Amersham Biosciences. A Wizard genomic DNA purification kit and pcCl plasmid vector were bought from Promega. A QuikChange™ site-directed mutagenesis kit was purchased from Stratagene. NotI, BsrGI, and XhoI were from New England BioLabs (Beverly, MA). Aminoethyl carbonate (1.5 mM/mL; specific activity, 3 Ci/mmol) was purchased from Amersham Biosciences and PerkinElmer Life Sciences. Di-n-butyl phthalate and di-isooctyl phthalate (BDH Chemicals, Poole, England) were blended 80:20 (v/v) to give a mixture of density 1.030 g/mL. Fura Red™ AM was from Molecular Probes. Fluorescein isothiocyanate (FITC)-, phycoerythin (PE)-, and PE-Cy5-conjugated anti-CD monoclonal antibodies (mAbs) and horse-radish peroxidase (HRP)-conjugated rabbit anti-sheep and anti-rabbit immunoglobulin antibodies were from Dako. PE-conjugated sheep anti-mouse immunoglobulin antibody was from Chemicon (Temecula, CA). Cy3-conjugated donkey anti-sheep IgG antibody and Cy2-conjugated donkey anti-rabbit IgG antibody were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Murine anti-human P2X7 receptor mAb (kindly provided by Drs. Gary Buell and Ian Chessell) (32) was purified from clone L4 and 2B hybridoma supernatants by chromatography on protein A-Sepharose Fast Flow and conjugated to FITC as described previously (33). Sheep anti-human P2X2 polyclonal antibody against a non-homologous extracellular epitope of the human P2X2 receptor has been described previously (29). Rabbit anti-rat P2X7 polyclonal antibody cross-reacting with human P2X7 has also been described previously (29). The base change is in bold and underlined: G946A forward, CCC GAA GAC TTT TAT CAG AGT C

**DNA Extraction—**Genomic DNA was extracted from peripheral blood using the Wizard genomic DNA purification kit according to the manufacturer's instructions.

**PCR and DNA Sequencing of PCR Products—**Twelve primer pairs were designed to amplify the 13 exons of human P2RX7 gene from genomic DNA (GenBank™ accession number NT_009775.8) (Table I).

**DNA Sequencing—**DNA and gel band purification kit were from Amersham Biosciences. A Wizard genomic DNA purification kit and pcCl plasmid vector were from Invitrogen. Ficoll-PaqueTM PLUS and a GFX™ PCR DNA and gel band purification kit were from Amersham Biosciences. A Wizard genomic DNA purification kit and pcCl plasmid vector were bought from Promega. A QuikChange™ site-directed mutagenesis kit was purchased from Stratagene. NotI, BsrGI, and XhoI were from New England BioLabs (Beverly, MA). Aminoethyl carbonate (1.5 mM/mL; specific activity, 3 Ci/mmol) was purchased from Amersham Biosciences and PerkinElmer Life Sciences. Di-n-butyl phthalate and di-isooctyl phthalate (BDH Chemicals, Poole, England) were blended 80:20 (v/v) to give a mixture of density 1.030 g/mL. Fura Red™ AM was from Molecular Probes. Fluorescein isothiocyanate (FITC)-, phycoerythin (PE)-, and PE-Cy5-conjugated anti-CD monoclonal antibodies (mAbs) and horse-radish peroxidase (HRP)-conjugated rabbit anti-sheep and anti-rabbit immunoglobulin antibodies were from Dako. PE-conjugated sheep anti-mouse immunoglobulin antibody was from Chemicon (Temecula, CA). Cy3-conjugated donkey anti-sheep IgG antibody and Cy2-conjugated donkey anti-rabbit IgG antibody were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Murine anti-human P2X7 receptor mAb (kindly provided by Drs. Gary Buell and Ian Chessell) (32) was purified from clone L4 and 2B hybridoma supernatants by chromatography on protein A-Sepharose Fast Flow and conjugated to FITC as described previously (33). Sheep anti-human P2X2 polyclonal antibody against a non-homologous extracellular epitope of the human P2X2 receptor has been described previously (29). Rabbit anti-rat P2X7 polyclonal antibody cross-reacting with human P2X2, has also been described previously (34). The Mini-Complete protease inhibitor mixture, and centrifuged at 8,000 g for 40 s. The upper layer (0.7 ml) from each tube and 0.7 ml from lysates were removed, and the amount of radioactivity was detected by Cerenkov counting.

**Be2+ Influx Measurements—**Mononuclear cells (4 x 10⁶) were incubated with Fura Red (1 μg/mL) for 30 min at 37 °C in HEPES-buffered NaCl medium. Cells were then washed once and labeled with appropriate FITC-conjugated anti-CD mAbs for 15 min. Cells were washed once and resuspended in 1.0 ml of HEPES-buffered KCl medium at 37 °C. All samples were stirred and temperature-controlled at 37 °C using a Time Zero module (Cytek, Fremont, CA). BaCl₂ (1.0 mM) was added followed 40 s later by addition of 1.0 mM ATP. Cells were analyzed at 2,000 events/s on a FACSCalibur flow cytometer (BD Biosciences) and were gated by forward and side scatter and by cell type-specific antibodies. The linear mean channel of fluorescence intensity (0–1023 channel) for each gated subpopulation over successive 5-s intervals was analyzed by WinMDI software and plotted against time. Due to the increased P2X7 function on macrophages, ethidium bromeid uptake in these cells (see Fig. 11) was acquired at a reduced voltage setting for FL-2 (ethidium fluorescence) as described previously (29).

**Pld Assay—**B lymphocytes (1 x 10⁶ cells/ml) were cultured at 37 °C in 5% CO₂ overnight in supplemented RPMI 1640 medium containing 10% FCS and 1 mM CaCl₂ or RPMI 1640 medium containing 10% fetal calf serum and 5 mM CaCl₂. 86RbCl (1.5 mM/mL; specific activity, 3 Ci/mmol) for 30 min at 37 °C in HEPES-buffered NaCl medium. Cells were then washed once and labeled with appropriate FITC-conjugated anti-CD mAbs for 15 min. Cells were washed once and resuspended in 1.0 ml of HEPES-buffered KCl medium at 37 °C. All samples were stirred and temperature-controlled at 37 °C using a Time Zero module. Ethidium (25 μM) was added followed 40 s later by addition of 1.0 mM ATP. Cells were measured at 1,000 events/s on a FACSCalibur flow cytometer and were gated by forward and side scatter and by cell type-specific antibodies. The linear mean channel of fluorescence intensity (0–255 channel) for each gated subpopulation over successive 5-s intervals was analyzed by WinMDI software and plotted against time.

**Samples were then incubated in the presence of ATP (0.5 mM) or phorbol 12-myristate 13-acetate (0.1 μM) for 15 min at 37 °C. Membrane lipids were extracted, and the level of phosphatidylbutanol was determined as described previously (17).**

**DNA Extraction—**Genomic DNA was extracted from peripheral blood using the Wizard genomic DNA purification kit according to the manufacturer's instructions.

**Pcr and DNA Sequencing of PCR Products—**Twelve primer pairs were designed to amplify the 13 exons of human P2RX7 gene from genomic DNA (GenBank™ accession number NT_009775.8) (Table I). These oligonucleotides were designed by using Primer3® and synthesized by PROLOG (Sydney, Australia). PCR amplifications (39 cycles of denaturation at 94 °C for 45 s, annealing at 58 or 55 °C for 20 or 30 s, and extension at 72 °C for 15 s) produced 12 fragments of the expected size. PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining. Amplified PCR products were sequenced using the Gfx PCR DNA and gel band purification kit. Using the AmpliTaq FS dye terminator cycle sequencing kit (Applied Biosystems), a fluorescence-based cycle sequencing reaction was performed to sequence the PCR products of P2X7, directly from both ends using specific primers. Sequencing electrophoresis was carried out on the ABI PRISM 377 DNA sequencer and analyzed using ABI PRISM sequencing analysis software (version 3.0) at the SUPAMAC Facility, Royal Prince Alfred Hospital (Sydney, Australia).

**Site-directed Mutagenesis—**The full-length clone of human P2X7, (GenBank™ accession number Y09561) was used in these studies, kindly provided by Dr. Gary Buell as a NotI-NotI insert in pcDNA3. hP2X7 was removed from pcDNA3 using a NotI-NotI digest and ligated into pCI, which is a cytomegalovirus-driven mammalian expression vector. Mutated 946G→A was introduced using overlap PCR (QuikChange site-directed mutagenesis kit) and the expression vector pcCl-pH2X, as a template. The P2RX7 point mutation was introduced using a QuikChange site-directed mutagenesis kit and primers described below consisting of the mutagenic codon flanked by sequences homologous to the wild type strand of the template. After digestion of the parental DNA with DpnI, intact mutation-containing synthesized DNA was transformed into competent Blue XL cells. All mutations were confirmed by sequencing. The base change is in bold and underlined: 5′-GTTT ACG CTG ATA AAA GTC GTC GGG; G946A reverse, CCC GAA GAC TTT TAT CAG AGT CTT TTC CTC AAC ATT G. See www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.
Transfection of HEK 293 Cells—HEK-293 cells were cultured in complete RPMI 1640 medium. The culture was negative for Mycoplasma as monitored by PCR every 2–3 months of culture. Full-length P2X (30 μg) or mutant P2X, cDNA in pc1 vector were incubated in serum-free Opti-MEM I medium for 5 min followed by incubation with LipofectAMINE 2000 reagent (30 μl, diluted with Opti-MEM I medium) for 20 min at room temperature. The solution was transferred into a nearly confluent monolayer of HEK-293 cells (5 × 10^6 in 9 ml of complete RPMI 1640 medium without antibiotics). After 40–44 h, cells were collected by mechanical scraping in complete RPMI 1640 medium.

Immunofluorescent Staining and Flow Cytometry—Mononuclear cell preparations (1 × 10^6/ml) from healthy or chronic lymphocytic leukemia (CLL) subjects or HEK-293 cells were incubated for 20 min at 30 °C with FITC-conjugated P2X, mAb (clone L4) plus PE-conjugated CD3 and PE-Cy5-conjugated CD19 mAbs in HEPES-buffered saline containing 10% group AB human serum. Cells were washed once and analyzed for P2X, expression on lymphocyte subpopulations gated for B lymphocytes (CD19+) or T lymphocytes (CD3+) or on HEK-293 cells gated by forward and side scatter. Dead cells were excluded by 7-aminoactinomycin D staining. For intracellular staining, HEK-293 cells were fixed with 1% paraformaldehyde for 15 min at 4 °C and labeled with FITC-P2X, mAb for 15 min in the presence of 0.1% saponin and 10% group AB human serum. Isotype control values for lymphocytes ranged from 2 ± 1 mean channels of fluorescence intensity and were subtracted from the values for each type.

Immunofluorescent Staining and Confocal Microscopy—Non-transfected, mock-transfected, and transfected HEK-293 cells were harvested and cultured on collagen-coated (50 μg/ml) glass coverslips in 48-well plates at 2.5 × 10^4 cells/well for 120 min. Cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and washed three times with PBS. Cells were blocked with 20% normal horse serum in PBS for 20 min before incubation with sheep anti-human or rabbit anti-antibody, P2X, polyclonal antibodies or preimmune serum diluted in PBS for 120 min. Cells were washed and incubated with Cy5-conjugated donkey anti-sheep or Cy2-conjugated donkey anti-rabbit IgG antibody diluted in PBS for 60 min. Washed cells were mounted on glass slides in glycerol gelatin mounting medium and visualized with a Leica TCS NT UV laser confocal microscope system.

Western Blotting—HEK-293 cells (5 × 10^6) transfected with wild type or R307Q mutant human P2RX7 cDNA were resuspended in digestion buffer containing Mini-Complete protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 750 mM e-amino-n-caproic acid, and 50 mM BisTris (pH 7.0). Cells were lysed with 1% n-dodecyl-β-D-maltoside at 4 °C followed by centrifugation at 20,000 × g for 15 min. The supernatants were collected and separated by 8–16% SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose membrane and blocked overnight in TTBS buffer (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5) containing 5% skim milk powder. The membrane was washed and incubated with a sheep anti-human or a rabbit anti-rat P2X, receptor polyclonal antibody (1:1,000) in TTBS buffer for 2 h. The membrane was washed and incubated with horseradish peroxidase-conjugated rabbit anti-sheep or swine anti-rabbit immunoglobulin antibody (1:5,000) for another 1 h. The horseradish peroxidase was then detected with the SuperSignal kit.

Electrophysiology—Oocytes from adult female Xenopus laevis were surgically removed and prepared as outlined previously (28). Stage 5 or 6 oocytes were injected with 50 nl of cRNA encoding wild type P2RX7 or mutant P2RX7 R307Q receptors (−1 μg/μl) and were stored at 18 °C for 2 days prior to experimentation. For two-electrode voltage clamp recordings, oocytes were impaled with two glass electrodes containing 3 M KCl and held at a membrane potential of −70 mV with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). Oocytes were continually perfused with ND96 solution (96 mM NaCl, 2 mM KCl, 0.1 mM CaCl2, 5 mM HEPES, pH 7.5) using a pump perfusion system. 100 μM ATP dissolved in bath solution was applied to the oocyte at 18 °C until the response reached a plateau or alternatively, if no response was observed, for ~1 min. Following ATP application, the oocyte was again perfused with bath solution, and the inward current trace was monitored until full recovery was observed.

RESULTS

A Single Nucleotide Polymorphism at Position 946 of the P2RX7 Gene—Both loss-of-function polymorphisms identified to date in the human P2RX7 gene result from single base substitutions in exon 13. A search was made for other single nucleotide polymorphisms by sequencing the other 12 exons of P2RX7 gene from genomic DNA of healthy and leukemic subjects. In two of 110 CLL patients and 10 of 310 healthy subjects, a heterozygous nucleotide substitution (946G→A) was found in exon 9, but no homozygous substitutions were observed (Table II). This substitution predicts a change of Arg946 to glutamine (R307Q) in the extracellular domain of the P2X, receptor. The overall allele frequency of this single nucleotide polymorphism was 0.014 in the Caucasian population when healthy subjects and CLL patients were considered as a single group (n = 420). Thus this mutant allele falls within the definition of a single nucleotide polymorphism since its prevalence is greater than 0.01 (1%) in the population. Of the total 12 subjects who were heterozygous for 946G→A, two were also heterozygous for other loss-of-function polymorphisms: one for 1513A→C and one for 1729T→A (Table II).

P2X, Expression and Function in Monocytes and Lymphocytes—Mononuclear preparations from healthy and CLL subjects with known residues at positions 307, 496, and 568 were preincubated with appropriate FITC-labeled mAbs, and ATP-induced uptake of ethidium into gated lymphocyte and monocyte subpopulations was measured as described previously (27, 33). Absent or very reduced P2X, function was found in all six R307Q heterozygous subjects who were tested. In contrast, mononuclear cells from subjects who were wild type at all three polymorphic positions showed robust P2X, function (Table II and Fig. 1). When the anti-human P2X, mAb (clone L4) was used in this study as described previously (33), the R307Q heterozygotes also showed decreased binding of this antibody (Table II).

ATP-induced 86Rb Efflux from Lymphocytes—The function of the P2X, channel/pore was measured by the ATP-induced efflux of isotopic Rb+ from lymphocytes (>98% purity) isolated from peripheral blood of the two subjects with CLL. In wild type lymphocytes the loss of 86Rb+ from the cells over 4 min followed first-order kinetics with a rate constant of 0.03 ± 0.01

TABLE I

| Region | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) | Product size (bp) |
|--------|-------------------------|--------------------------|------------------|
| Exon 1 | tcgattgctgcatcaggtcag | ccaagtcgtttcacttttgca | 315              |
| Exon 2 | ggtctgatactctggaggaag | cctgctgcatcttct        | 379              |
| Exon 3 | tgcagctgcatcaggttcctc | ccctgcaagctgattat      | 230              |
| Exon 4 | tgcagtgcctgcatcacaat | gttgcactctttggtgatt    | 241              |
| Exon 5 | tggacctgacagctttccag | ggtggtagttagtaatgtgcc  | 299              |
| Exon 6 | ttcagctgctgtagttggtg | agagctcgtgctgctttcag  | 239              |
| Exon 7 | gcctctgtgcttgcttgctat | tggactctttcccacaccact  | 300              |
| Exon 8 | tgcagctgcatcaggttgc | ctgctgcatgtagttagtaatct | 300          |
| Exon 9 | gcccacccagctgtttcaggag | gttgcagttgtagttatccc  | 279              |
| Exons 10 and 11 | tagacccagcagctgattac | ccacacctgctgcttggaagctg | 399          |
| Exon 12 | ggccacgctgtgctgctttcct | tggacctctttcccacaccact | 300          |
| Exon 13 | gagacctgtagccacgtggct | gacacctgtagccacgtggct | 579              |


**Fig. 1.** ATP-induced ethidium uptake curve in mononuclear cell subsets from healthy wild type and R307Q heterozygous subjects. 2 × 10⁶ cells prelabeled with appropriate FITC-conjugated cell-specific mAb were incubated in 1 ml of KCl medium at 37°C. Ethidium bromide (25 μM) was added followed 40 s later by 1 mM ATP. The mean channel of cell-associated fluorescence intensity was measured at 5-s intervals for B lymphocytes (gated CD19⁺), T lymphocytes (gated CD3⁺), natural killer (NK) cells (gated CD16⁺), and monocytes (gated CD14⁺).

**TABLE II**

| Genotype | P2X7 expression (mean channels of fluorescence intensity) | ATP-induced ethidium uptake at 5 min |
|----------|----------------------------------------------------------|-------------------------------------|
| 946 G/A  | A/A T/T                                                  | 2.1 1.7                             |
| 1513 A/A | A/A T/T                                                  | 3.6 5.1                             |
| 1729 A/A | A/A T/T                                                  | 10.0 ± 5.0 7.1 ± 3.5                |
| Healthy subjects |                                | 615 0 2010 2.951 ± 2.311             |
| N1 G/A   | A/A T/T                                                  | 6.1 4.3                             |
| N2 G/A   | A/A T/T                                                  | 2.1 1.5                             |
| N3 G/A   | A/C T/T                                                  | 2.5 3.8                             |
| N4 G/A   | A/A T/T                                                  | 1.0 1.8                             |
| N5 G/A   | A/A T/T                                                  | 3.0 4.8                             |
| CLL subjects |                                | 615 0 2010 2.951 ± 2.311             |
| C1 G/A   | A/A T/A                                                  | 2.1 1.7                             |
| C2 G/A   | A/A T/T                                                  | 3.6 5.1                             |
| Healthy wild type subjects |                                | 4,013 ± 2,010 2,951 ± 2,311           |

*min⁻¹ (range, 0.01–0.05) in the absence of ATP and 0.34 ± 0.04 min⁻¹ (range, 0.24–0.50) in the presence of ATP (Fig. 2). In subject C1 who was heterozygous for both R307Q and I568N, ATP-induced Rb⁺ efflux was absent, while in subject C2, who was heterozygous for R307Q but wild type for E496A and I568N, the rate constant was reduced to 0.27 min⁻¹. Attempts to measure Rb⁺ efflux from lymphocytes of healthy subjects were complicated by a 5–10% monocyte admixture in the preparation, and a flow cytometric approach was adopted to further study P2X7 channel fluxes.

**ATP-induced Ba²⁺ Influx into Lymphocyte Subsets**—The permeability of the P2X7 channel/pore was also studied by two-color flow cytometry in which the influx of Ba²⁺ was measured in T lymphocytes identified by appropriate FITC-conjugated mAb. Fig. 3 shows the ATP-induced uptake of Ba²⁺ into T lymphocytes loaded with Fura Red whose fluorescence emission measured by flow cytometry decreases on chelating this divalent cation as it enters the cell. The rate of Ba²⁺ uptake into lymphocytes from subject N1 who was heterozygous for R307Q was markedly decreased compared with a wild type subject (Fig. 3). Absence of ATP-induced Ba²⁺ uptake was found in T lymphocytes from subject C1 who was double heterozygous for R307Q and I568N (Fig. 3). Similar results were obtained for T lymphocytes as well as natural killer cells and B cells from other heterozygous subjects (data not shown).

**ATP-stimulated PLD Activity Was Impaired in Subject Double Heterozygous for R307Q/I568N Polymorphism—**A major downstream effect of P2X7 receptor activation is stimulation of PLD activity demonstrated both in macrophages (16) and CLL lymphocytes (17). Lymphocytes from a CLL patient (>98% purity) were incubated with or without ATP, and the PLD activity was measured by the transphosphatidylation reaction (17). Fig. 4 shows that lymphocytes from three CLL patients who were wild type for R307/E496/I568 demonstrated strong ATP-stimulated PLD activity, while lymphocytes from subject C1 who was double heterozygous for R307Q/I568N failed to
show ATP-stimulated PLD activity despite the similar level of total PLD activity stimulated by phorbol 12-myristate 13-acetate.

Expression and Function of Arg^{307} to Gln (R307Q)-mutated P2X_{7} in HEK-293 Cells—cDNA for wild type P2X_7 or P2R7 carrying the 946G→A mutation was transfected into HEK-293 cells, and the expression and function of the receptor was measured. At 44 h after transfection, an aliquot of the cells was lysed, and proteins were separated by SDS-PAGE. Western blotting using a sheep anti-rat P2X_7 polyclonal antibody (data not shown). Despite strong P2X_7 protein expression on the cell surface, the P2X_7 receptor was detected. Untransfected HEK-293 cells were used as control. Similar results were obtained by using the rabbit anti-rat P2X_7 antibody (data not shown).

A mutation was injected into oocytes, and the channel function of the receptor was measured. At 44 h after injection, an aliquot of the cells was lysed, and proteins were separated by SDS-PAGE. Western blotting using a sheep anti-rat P2X_7 polyclonal antibody (data not shown). Despite strong P2X_7 protein expression on the cell surface, the P2X_7 receptor was detected. Untransfected HEK-293 cells were used as control. Similar results were obtained by using the rabbit anti-rat P2X_7 antibody (data not shown).
FIG. 6. Confocal microscope images of P2X<sub>7</sub> expression in HEK-293 cells. HEK-293 cells were transiently transfected with pCI Vector (Mock); wild type (WT), R307Q, or I568N mutant P2X<sub>7</sub> cDNA as indicated. Cells were fixed and incubated with preimmune serum or sheep anti-human P2X<sub>7</sub> antibody and subsequently with Cy3-conjugated anti-sheep IgG antibody before analysis by confocal microscopy. Similar results were observed using the rabbit anti-rat P2X<sub>7</sub> antibody (data not shown). Preimmune serum showed no binding.

FIG. 7. BzATP-induced ethidium<sup>+</sup> uptake into transfected HEK-293 cells. Cells were transiently transfected with pCI Vector (Mock), wild type, or R307Q-mutated P2X<sub>7</sub> cDNA as indicated. Cells were washed and resuspended in 1 ml of NaCl medium. Ethidium<sup>+</sup> (25 μM) was added followed 40 s later by BzATP. The mean channel of cell-associated fluorescence intensity was measured at 5-s intervals for gated HEK-293 cells. 150 s after addition of BzATP, the area under the ethidium uptake curve was calculated and normalized. Results were presented as mean ± S.D. (n = 3).

Failure of L4 Monoclonal Antibody to Bind Arg<sup>307</sup> to Gln<sup>312</sup>—The study of P2X<sub>7</sub> has been greatly assisted through the development of an anti-P2X<sub>7</sub> mAb (clone L4) that binds to the extracellular domain of P2X<sub>7</sub> and blocks receptor function (32, 33). Moreover this mAb binds not only to cell surface P2X<sub>7</sub> but also to the far larger intracellular pool of receptor function (32, 33). Moreover this mAb binds not only to cell surface P2X<sub>7</sub> but also to the far larger intracellular pool of receptor function (32, 33). However, at 44 h after transfection of R307Q-mutated P2X<sub>7</sub> into HEK-293 cells, anti-human P2X<sub>7</sub> mAb (clone L4) failed to bind either to cell surface P2X<sub>7</sub> or to intracellular P2X<sub>7</sub> (Fig. 9). A different anti-P2X<sub>7</sub> mAb (clone B2), which also binds to the extracellular domain of P2X<sub>7</sub> but without affecting its function (27), was chosen to examine the surface expression level. This B2 mAb bound avidly to wild type P2X<sub>7</sub> expressed on the surface of transfected HEK-293 cells, and binding of this mAb saturated between 1 and 10 μg/ml (Fig. 10). The B2 mAb also showed binding to R307Q mutant P2X<sub>7</sub> receptor expressed on the surface of transfected HEK-293, but the binding was of lower affinity compared with binding to wild type P2X<sub>7</sub>. Mock-transfected cells failed to bind the B2 mAb.

Absence of Macrophage P2X<sub>7</sub> Function in Double Heterozygotes—Differentiation of monocytes into macrophages increases the expression and function of P2X<sub>7</sub> by manyfold (35). Peripheral blood monocytes were cultured for 7 days with interferon-γ added for the final 24 h, and the P2X<sub>7</sub> function was measured by ATP-induced ethidium<sup>+</sup> uptake into the CD14<sup>+</sup> macrophage population. Macrophages isolated from healthy subjects who were wild type for R307, E496, and I568 showed at least 10-fold increases of surface expression and function of P2X<sub>7</sub> over values for monocytes (12, 27, 29). Although subjects heterozygous for R307Q showed nearly absent P2X<sub>7</sub> function in monocytes, their P2X<sub>7</sub> function was partially restored in macrophages to values 10–40% of wild type (Fig. 11). However, macrophages from subject N1 who was also heterozygous for R307Q failed to show any P2X<sub>7</sub> function possibly because of a second unidentified genetic defect (Fig. 11). Two subjects were double heterozygous for loss-of-function polymorphisms, one with R307Q combined with the trafficking-defective I568N allele (subject C1), while the second subject (N3) combined R307Q with the E496A polymorphism. Macrophages grown for 7 days from one of these double heterozygous subjects also showed a total absence of P2X<sub>7</sub> function when assayed multiple times (Fig. 11, subject C1).

DISCUSSION

The main finding in this study is that the single nucleotide polymorphism altering the positively charged Arg<sup>307</sup> to uncharged Gln in the extracellular domain of the P2X<sub>7</sub> receptor results in a complete loss of function. Positively charged and conserved amino acid residues such as arginine and lysine in

FIG. 8. ATP-invoked current in oocytes. Oocytes from adult female X. laevis were injected with 50 nl of cRNA encoding wild type P2RX7 (A) or mutant P2RX7 R307Q receptors (B) (−1 μg/μl) and were stored at 18 °C for 2 days prior to experimentation. For two-electrode voltage clamp recordings, oocytes were impaled with two glass electrodes containing 3 mol KCl and held at 18 °C at a membrane potential of −70 mV. 100 μM ATP dissolved in bath solution was applied to the oocyte as indicated. One representative experiment of four or five is shown.

The study of P2X<sub>7</sub> has been greatly assisted through the development of an anti-P2X<sub>7</sub> mAb (clone L4) that binds to the extracellular domain of P2X<sub>7</sub> and blocks receptor function (32, 33). Moreover this mAb binds not only to cell surface P2X<sub>7</sub> but also to the far larger intracellular pool of receptor function (32, 33). However, at 44 h after transfection of R307Q-mutated P2X<sub>7</sub> into HEK-293 cells, anti-human P2X<sub>7</sub> mAb (clone L4) failed to bind either to cell surface P2X<sub>7</sub> or to intracellular P2X<sub>7</sub> (Fig. 9). A different anti-P2X<sub>7</sub> mAb (clone B2), which also binds to the extracellular domain of P2X<sub>7</sub> but without affecting its function (27), was chosen to examine the surface expression level. This B2 mAb bound avidly to wild type P2X<sub>7</sub> expressed on the surface of transfected HEK-293 cells, and binding of this mAb saturated between 1 and 10 μg/ml (Fig. 10). The B2 mAb also showed binding to R307Q mutant P2X<sub>7</sub> receptor expressed on the surface of transfected HEK-293, but the binding was of lower affinity compared with binding to wild type P2X<sub>7</sub>. Mock-transfected cells failed to bind the B2 mAb.

The study of P2X<sub>7</sub> has been greatly assisted through the development of an anti-P2X<sub>7</sub> mAb (clone L4) that binds to the extracellular domain of P2X<sub>7</sub> and blocks receptor function (32, 33). Moreover this mAb binds not only to cell surface P2X<sub>7</sub> but also to the far larger intracellular pool of this receptor when cells are permeabilized to allow access of the
the extracellular domain of P2X receptors usually contribute to the binding of the negatively charged phosphate groups of ATP (25, 30, 31). Therefore, substitution of these residues to less positively charged, uncharged, or negatively charged residues effectively abolishes the binding of agonists. Arginine in the homologous position of the extracellular domain is conserved in all members of the P2X receptor family. Evidence from the study of the residue homologous to Arg307 in both P2X1 (Arg305) and P2X2 (Arg304) strongly suggests that this conserved residue is critical for activation by ATP (30, 31). Mutation of Arg305 in human P2X7 to the less positively charged lysine reduced the ATP-evoked channel peak current to one-quarter that of wild type, while mutating Arg305 to the mildly hydrophobic alanine completely abolished the ATP-evoked current (30). In the rat P2X7 receptor, Arg307 substitution to lysine or alanine reduced the ATP sensitivity by about 10- and 1000-fold, respectively (31). These data suggest that Arg307 forms part of an ATP binding pocket that probably also includes Lys311 since mutation of the latter residue to alanine also abolishes P2X7 function (25). This binding site may also include contributions from other positive residues since mutation of the conserved Lys309 and Arg292 in the P2X1 receptor either abolished function or reduced the potency of ATP binding (30). However, perhaps due to the fact that ATP binding to P2X7 is 2 orders of magnitude

Fig. 9. Flow cytometric histograms of P2X7 expression in HEK-293 cells using clone L4 mAb. HEK-293 cells were transiently transfected with wild type (top panels) or R307Q-mutated (bottom panels) P2X7 cDNA. Non-permeabilized cells (left panels, surface P2X7 expression) and fixed and permeabilized cells (right panels, intracellular P2X7 expression) were labeled with FITC-conjugated anti-P2X7 mAb (clone L4) (solid line) or isotype control mAb (shaded line), and the level of P2X7 expression was determined by flow cytometry. One representative experiment of four is shown.

Fig. 10. Saturation curve of clone B2 mAb. HEK-293 cells were transiently transfected with pCI Vector (Mock), wild type, or R307Q-mutated P2X7 cDNA as indicated. Cells were labeled with mouse anti-human P2X7 mAb (clone B2) for 20 min at room temperature and washed twice before incubation with PE-conjugated sheep anti-mouse immunoglobulin antibody. Cells were washed once, and the mean channel of cell-associated fluorescence was measured by flow cytometry. One representative experiment of two is shown.

Fig. 11. ATP-induced ethidium+ uptake in monocyte-derived macrophages. Monocyte-derived macrophages (activated with interferon-γ) from a wild type subject or R307Q heterozygotes (see Table II) were labeled with FITC-conjugated anti-CD14 mAb and suspended in KCl medium at 37 °C. Ethidium+ (25 μM) was added followed 40 s later by the addition of 1 mM ATP (arrow). The mean channel of cell-associated fluorescence was measured by time-resolved flow cytometry. The voltage setting for ethidium was reduced to gain full scale of uptake increase. Basal ethidium+ uptake measured in the absence of ATP is shown.
A Polymorphism within the ATP-binding Site of P2X7

Weaker than to P2X1, modification of any of the critical residues of P2X7 causes total loss of nucleotide binding (25). Further insight into the ATP-binding site of P2X7 is suggested by secondary structure predictions based on homology with the known structure of class II aminoacyl-tRNA synthetases (24, 36). This indicates a conserved structure of six β-pleated sheets between Phe188 and Val221, and further modeling reveals that Lys193 and the cluster of charged groups including Arg307 and Lys311 are located within this subdomain of the extracellular loop. Whether the ATP-binding site is formed at the interface between adjacent monomers in the assembled channel/pore or lies entirely within each monomer remains uncertain.

Two other polymorphisms, E496A and I568N, have been characterized in healthy and leukemic human subjects and shown to cause loss of function of the P2X7 receptor. Homozygosity for E496A produces the same amount of P2X7 protein with markedly reduced pore function, while the heterozygous state gives cells with half the pore function of cells with wild type P2X7 protein (27). Recent studies have found that the E496A polymorphism does not affect the electrophysiological phenotype of the P2X7 channel in transfected oocytes and HEK-293 cells (37), and we have confirmed these findings.3 However, the E496A functional pore defect can be overcome partially when the density of these receptors on the cell surface is massively increased following differentiation of monocytes to macrophages (27). A second loss-of-function polymorphism, I568N, has been described within a trafficking motif in the carboxyl terminus of P2X7 receptor (28) that blocks receptor function by preventing the receptor trafficking to the cell surface (29). Large amounts of I568N-mutated P2X7 protein is found in the intracellular location, but none is found on the cell surface of transfected HEK-293 cells. Monocytes from subjects heterozygous for I568N have nearly absent P2X7 function, while macrophages from these same subjects show partial P2X7 function presumably due to P2X7 up-regulation from the normal Ile568 allele. However, not all individuals with low P2X7 function can be explained by these two polymorphisms, both of which lie in exon 13. Therefore, an extensive search of the other 12 exons of the P2RX7 gene was conducted to find other loss-of-function polymorphisms. The mutation reported in this study, R307Q, has not been reported in any public polymorphism data base, and it is the third polymorphism found to cause loss-of-function polymorphisms. The mutation reported in this study, R307Q, has not been reported in any public polymorphism data base, and it is the third polymorphism found to cause loss-of-function of the human P2X7 receptor with an allele frequency of 0.014 in the Caucasian population. Our study demonstrates that subjects who are double heterozygous (compound heterozygotes) show the most profound loss of P2X7 function. Thus subject C1, who was double heterozygous for R307Q/I568N, failed to show any functional P2X7 in all our functional assays of all mononuclear cell types including macrophages (Figs. 1, 2, 3, 4, and 11).

Fig. 9 shows that the R307Q mutant P2X7 receptor expressed in HEK-293 cells fails to bind the anti-human mAb (clone L4). Buell and colleagues (32) and our laboratory (33) have shown that the L4 mAb binds to an unknown extracellular epitope and blocks ATP activation of this receptor by acting as a functional antagonist. Western blotting showed robust binding by forming part of the recognition epitope; this in turn suggests that L4 mAb blocks P2X7 function by steric hindrance at the ATP binding pocket. In contrast, Arg307 is important but not essential for binding of the B2 mAb, and this mAb does not block P2X7 function (27).

Dilatation of the P2X7 channel to form a pore under physiological conditions is recognized as a unique feature of the P2X7 receptor. However, opening of the ionic channel and formation of the pore are two distinct processes (28, 38) since the carboxyl-terminal truncated P2X7 receptor retains channel activity but lacks pore formation. In this study, monocytes and macrophages from subjects who were heterozygous for R307Q showed variable reduction of ATP-induced ethidium uptake to values of 0–50% of wild type (Figs. 1 and 11). The ATP-induced Ba2+ influx into lymphocytes was also greatly reduced when subsets were identified using two-color flow cytometry (Fig. 3). However, the fluorometric methods used in this study are not sensitive enough to distinguish the channel and pore function of the P2X7 receptor. Nevertheless our electrophysiological study (Fig. 8) clearly shows that the R307Q polymorphism abolishes P2X7 channel function as expected by the loss of ATP binding.

Our data are consistent with a role for the P2X7 receptor in the innate immune response against obligate intracellular pathogens. There is evidence that activation of the P2X7 receptor is required for fusion of lysosomes with the phagosomes of the macrophage, which contains engulfed mycobacteria or chlamydiae. The formation of a phagolysosome results in the subsequent killing of these pathogens by a process that is dependent on PLD stimulation (39–42). Thus the results in Fig. 4 are significant since subject C1 who was double heterozygous for R307Q/I568N showed no ATP-stimulated PLD activity, while the same subject (Fig. 11) showed total absence of P2X7 function in macrophages. Moreover we have recently shown that ATP-induced killing of intracellular mycobacteria is impaired in macrophages from subjects with low P2X7 function due to homozygosity for the E496A polymorphism (43). Our data raise the possibility that low or absent P2X7 receptor function due to inherited polymorphisms of this receptor may be a genetic susceptibility factor in a range of infections as diverse as tuberculosis, toxoplasma, or chlamydia. Individuals who carry two loss-of-function polymorphisms (compound heterozygotes) have the highest susceptibility to these infections by intracellular pathogens because of the central role of macrophages in innate immunity. It is likely that more polymorphisms affecting function will be found within the coding and non-coding regions of the P2RX7 gene, and their clinical associations will be the subject of further study.

Acknowledgments—We thank Dr. Gary Buell and Dr. Ian Chessel for providing mAb and Dr. Diana Williams for blood collection.

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A Polymorphism within the ATP-binding Site of P2X7
An Arg\textsuperscript{307} to Gln Polymorphism within the ATP-binding Site Causes Loss of Function of the Human P2X\textsubscript{7} Receptor

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J. Biol. Chem. 2004, 279:31287-31295.
doi: 10.1074/jbc.M313902200 originally published online April 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313902200

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