Point mutations confer loss of ATP-induced human $P2X_7$ receptor function

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Abstract Residues considered essential for ATP binding to the human $P2X_7$ receptor ($hP2X_7R$) were investigated. HEK293 cells or *Xenopus* oocytes were transfected with wild-type or site-directed mutants of $hP2X_7R$ constructs and channel/pore activity measured in the presence of ATP or 2′,3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP). Barium uptake and ethidium influx into HEK293 cells were abolished in cells expressing K193A and K311A mutants, and were partially reduced in cells expressing mutant P210A. K193A and K311A mutations also completely abolished responses to ATP and BzATP in *Xenopus* oocytes as measured by electrophysiology. These results indicate that K193 and K311 are essential residues in ATP binding in the $hP2X_7R$. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: $P2X_7$; Channel function; Cytolytic pore; Point mutation

1. Introduction

Extracellular ATP is a ubiquitous signaling molecule that exerts its effects by binding to a family of P2X receptors, designated $P2X_{1-7}$, which are fast, ligand-gated cation channels. These subtypes exhibit extensive homology (30–40%) but differ considerably in the length of their carboxy-termini. The long intracellular C-terminus is unique to $P2X_7$, and is thought to confer time-dependent dilation of the channel to form a pore [1]. Three polymorphisms have been identified in the $P2X_7$ gene that alters the primary structure of the receptor [2]. One of these at position 496 in the carboxy-terminal tail leads to loss of the permeability properties of $P2X_7$ for both small inorganic cations and large organic cations [3]. The functional effects of the common polymorphisms at positions 155 and 348 have not been reported.

There have been few studies of the residues in the extracellular domain that are critical for agonist binding to $P2X_7$. Positively charged amino acids, such as lysine, have been shown to be important in ATP binding by interacting with the phosphate groups of the ATP molecule. In the extracellular loop of the P2X receptors, there are seven conserved lysines. Mutation at two of these in $hP2X_1$, K70 and K309, decreased the potency of ATP by many orders of magnitude [4]. In $rP2X_2$, mutation at K69 and K188 also decreased the potency of ATP, and mutation at K308 abolished the response [5]. The exact location of the ATP binding pocket in human $P2X_7$ receptor ($hP2X_7R$) is uncertain, though many studies of the $P2X_7$ purinoceptor indicate a cooperative process in pore opening, suggested by Hill coefficients of approximately two observed for mast cells and thymocytes [6,7], and $hP2X_7R$ expressed in *Xenopus* oocytes [8] and studies indicate that the increment in permeant flux shows a sigmoid dependence on ATP concentration. This cooperative effect of ATP in stimulating permeant influx is consistent with ATP binding to multiple sites on an oligomeric channel to initiate opening. In one study on $P2X_7$ receptors, residue 214 was associated with an ATP binding site based on marked species differences in agonist dissociation rates between rat, mouse and human $P2X_7$ [9].

In the present study de novo protein design was utilized to determine if two of the seven conserved lysine residues in the extracellular loop of $P2X_7$ were critical sites for nucleotide binding. A proline residue downstream of the first identified lysine was also targeted to examine agonist potency at the putative binding site. Furthermore, two polymorphic changes, H155Y and A348T, identified in human population studies of $P2X_7$ [2], also were tested. Since truncation of the carboxy-terminal tail of the rat $P2X_7$ receptor ($rP2X_7R$) inhibits pore but not channel properties [10], a C-terminal truncated $hP2X_7R$ was investigated.

2. Materials and methods

2.1. Materials

ATP, 2′,3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP), ethidium bromide, barium chloride, RPMI 1640 medium, D-glucose, BSA (bovine serum albumin), and FluoroTag FITC Conjugation Kit 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); Cy2-conjugated anti-mouse 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); and mMessage mMachine kit mRNA from Ambion (Austin, TX, USA).

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2.2. Tissue culture

HEK293 cells were transfected with cDNAs encoding human or one of six mutant constructs of $P2X_7$ receptors using Lipofectamine 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.

2.3. Site-directed mutagenesis

The full-length clone of $hP2X_7$ (GenBank accession number Y09561) was prepared as described [3]. The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) was used according to the manufacturer's instructions to perform point mutations in the $hP2X_7Rs$.

Primer sequences for site-directed mutagenesis are as follows:

K193A forward: 5′-GCCGAAAACTTCACTGTGCTCATC**GC**-GAACAATATCGAC-3′

K193A reverse: 5′-GTCGATATTGTTC**GC**GATGAGCACAGT-GAAGTTTTCGGC-3′

P210A forward: 5′-CACCACGAGAAACATCCTG**G**CAGGTTT-AAACATCAC-3′

P210A reverse: 5′-GTGATGTTTAAACCTG**C**CAGGATGTTTC-TCGTGGTG-3′

K311A forward: 5′-GAAACGGACTCTGATA**GC**AGTCTTCG-GGATCCGTTTTG-3′

K311A reverse: 5′-CAAAACGGATCCCGAAGACT**GC**TATCA-GAGTCCGTTTC-3′

H155Y forward: 5′-CCGGAAGGTGTGTAGTGT**A**TGAAGGG-AACCAGAAGACC-3′

H155Y reverse: 5′-GGTCTTCTGGTTCCCTTCAT**A**CACTACA-CACCTTCCGG

A348T forward: 5′-CTTCGGTCTGGCCA**C**TGTGTTCATCGAC

A348T reverse: 5′-GTCGATGAACACAG**T**GGCCAGACCGA-AG

E496A forward: 5′-GGTGCCTGGAGG**C**GCTGTGCTGCCGG

E496A reverse: 5′-CCGGCAGCACAGC**G**CCTCCAGGCACC

Base changes introducing the mutations are in bold type. Truncated $hP2X_7$ (1–415) in pcDNA 3 (Invitrogen) was provided by Dr. G. Buell. Site-directed mutations were confirmed by sequence analysis.

2.4. Xenopus oocyte in vitro RNA preparation

The in vitro RNA preparation protocol used was as described [11].

2.5. Measurement of ATP-evoked currents in Xenopus oocytes by two-electrode voltage clamp

Oocytes were prepared from adult female *Xenopus laevis* (Disa Exporters, Somerset West Cape, South Africa) using standard procedures. Stage 5 or 6 oocytes were injected with approximately 50 ng of cRNA; $hP2X_7$ wild-type and human mutants K193A or K311A. Injected oocytes were stored at 18°C for 2 days before functional assay. Oocytes were impaled with two glass electrodes containing 3 M KCl and were clamped at a membrane potential of −70 mV using an Axoclamp 2B amplifier. Oocytes were perfused (2 ml/min) with a low divalent ND96 solution (96 mM NaCl, 2 mM KCl, 0.1 mM $BaCl_2$, 5 mM HEPES, pH 7.5). ATP or BzATP was applied for 15 s or until a peak current was recorded. Any additional doses of ATP were applied after a 20 min washout period. All electrical recordings were carried out at room temperature (20–22°C).

2.6. $P2X_7$ transfection of HEK293 cells

HEK293 cells do not natively express any of the known P2X receptor subtypes at sub-confluence, allowing for the expression of the $P2X_7R$ as a homomeric receptor in this cell type. However, on reaching over-confluence HEK293 cells express $P2X_{1-6}$ [12] as well as $P2X_7$ (unpublished results). Truncated, germline and mutant pCI-P2X7 plasmids were transfected into HEK293 cells as described [3].

2.7. Immunofluorescent staining

Immunofluorescent staining and confocal microscopy of HEK293 cells incubated on collagen-coated glass coverslips was performed as described [13] using a $P2X_7$ polyclonal antibody [14]. Anti-human $P2X_7R$ monoclonal antibody clones L4 and B2 were prepared as described [3,15].

2.8. Ethidium influx measurement by flow cytometry

Flow cytometry was used to quantitate ethidium bromide uptake as described [16].

3. Results

3.1. Surface expression of $P2X_7$ constructs in transiently transfected HEK293 cells

Confocal microscopy showed wild-type $P2X_7$ (Fig. 1a) and mutants P210A (Fig. 1d), K193A (Fig. 1e), K311A (Fig. 1f) expressed equally strongly on the surface of HEK293 cells where they were labeled using Cy3 bound to a polyclonal $P2X_7$ antibody. Negative controls included untransfected cells (Fig. 1b) and wild-type in the presence of 10 μM adsorption control peptide epitope (Fig. 1c). The extracellular epitope on

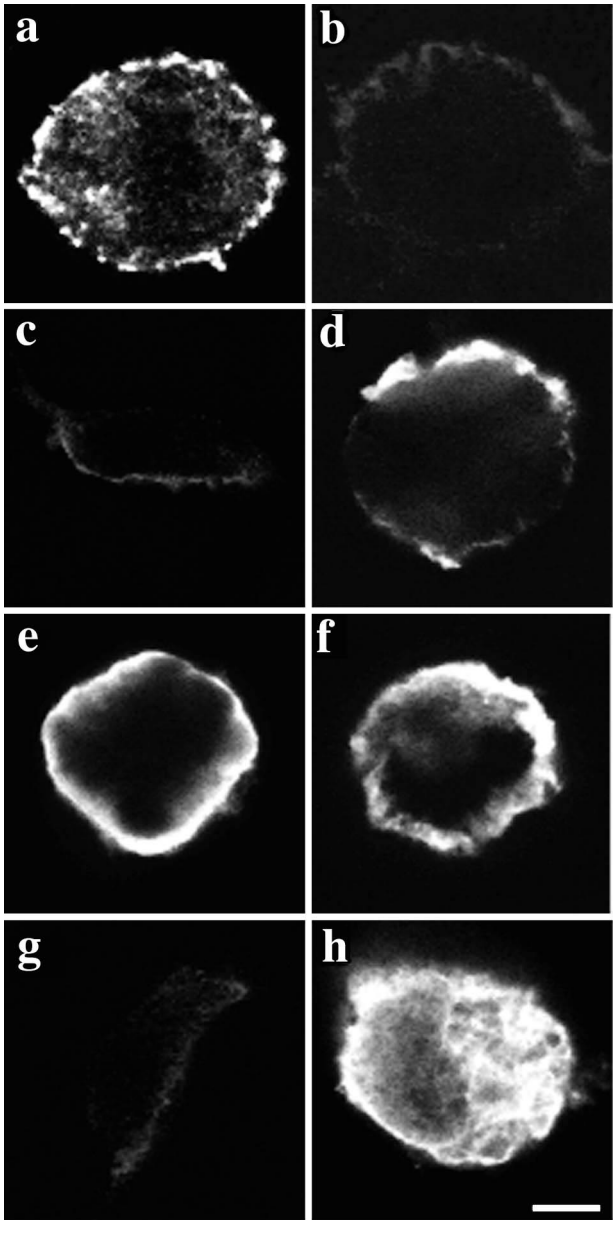

Fig. 1. Confocal microscope images of human and mutant $P2X_7R$ expression on the surface of HEK293 cells. HEK293 cells transiently transfected with either (a) wild-type, (b) untransfected control, (c) wild-type epitope block, (d) P210A, (e) K193A, (f) K311A, (g) truncated or (h) permeabilized cells with truncated $P2X_7$ cDNA were labeled with antibody against $P2X_7$ and subsequently with Cy3-conjugated anti-rabbit IgG antibody. Non-transfected cells were included as a negative control and showed no staining. The calibration bar is 4 μm.

the C-terminal truncated protein was unlabeled (Fig. 1g) unless cells were permeabilized (Fig. 1h) indicating the truncated mutant could not insert into the cell membrane. Identical results were obtained using anti-$P2X_7$ monoclonal antibodies (data not shown).

3.2. Functional analysis of pore formation of $P2X_7R$ constructs by ATP-induced uptake of ethidium and Ba^{2+}

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 untransfected HEK293 cells. ATP produced an eight-fold increase in the rate of ethidium influx in cells transiently transfected with wild-type $hP2X_7$, H155Y and A348T cDNA. ATP-induced ethidium uptake was abolished in cells expressing mutants K193A or K311A receptors (see Fig. 2). Cells expressing mutant P210A elicited a reduced response compared with wild-type. Fluorometry was used to examine the entry of Ba^{2+} via the $P2X_7$ channel in cells loaded with Fura-2 [17]. ATP-induced Ba^{2+} influx was measured in cells transfected with the $P2X_7$ constructs and incubated in both HEPES-buffered KCl buffer and sucrose buffer. Identical results were obtained for all constructs using fluorometry as for flow cytometry. ATP was unable to induce Ba^{2+} uptake in cells transfected with the K193A and K311A mutants, with the P210A mutant attenuating Ba^{2+} uptake compared to wild-type $hP2X_7R$, in HEPES-buffered KCl buffer. Both wild-type and P210A $hP2X_7R$ demonstrated relatively increased Ba^{2+} uptake in sucrose buffer due to the removal of extracellular Cl^- ions, as previously reported for wild-type $P2X_7R$ [18]. However, neither the K193A or K311A mutants showed Ba^{2+} uptake in the Cl^--free sucrose buffer (data not shown).

3.3. Expression of $hP2X_7$ mutants in Xenopus oocytes

As expected from $rP2X_7$ [11], expression of wild-type $hP2X_7$ resulted in robust ATP-activated inward current while sham-injected oocytes gave no response. Introduction of K193A and K311A mutations completely abolished receptor responses to ATP (Fig. 3).

In oocytes expressing wild-type $hP2X_7R$, BzATP elicited greater responses than ATP and the recovery of current to baseline following BzATP was significantly longer than for ATP (BzATP 5.8 ± 1.6 min, $n = 3$ vs. ATP 2.0 ± 1.1 min, $n = 4$). BzATP, like ATP, was without effect on oocytes injected with K193A and K311A hP2X7R cRNA (Fig. 3).

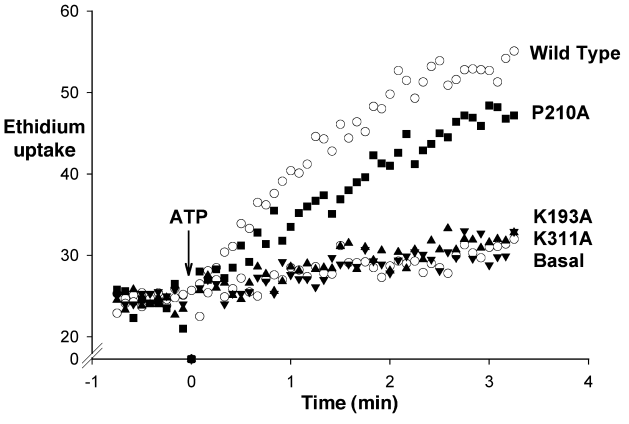

Fig. 2. ATP-induced ethidium uptake into transiently transfected HEK293 cells. Mean channel fluorescence intensity measured over 5 s intervals for wild-type, P210A, K193A or K311A $P2X_7$.

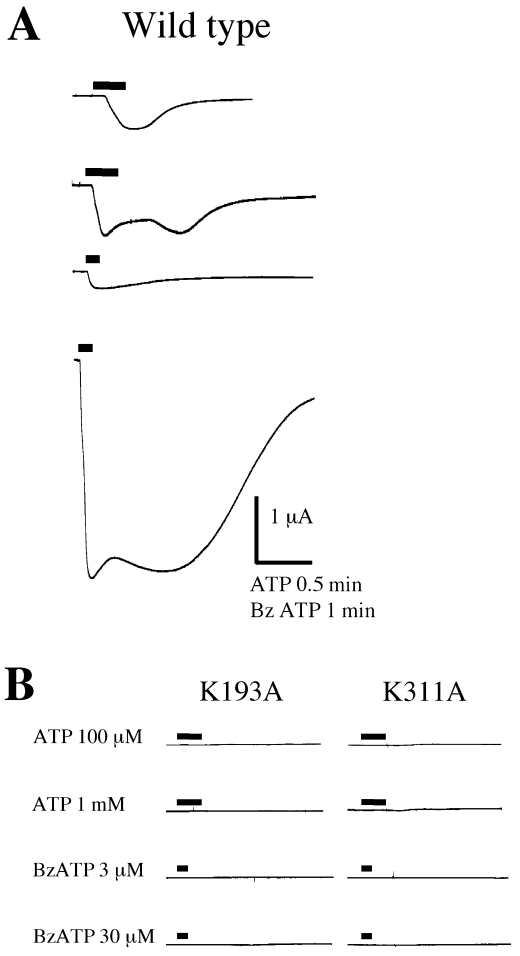

Fig. 3. Comparison of response to ATP and BzATP in wild-type and mutant $hP2X_7Rs$ expressed in *Xenopus* oocytes. Application of 100 μM ATP or 3 μM BzATP induced a rapid inward current in wild-type $hP2X_7$-injected oocytes, with sensitivity to BzATP significantly greater than to ATP. No agonist-induced current was observed in oocytes injected with K193A or K311A $hP2X_7$ cRNA. Saturation of the $P2X_7R$ constructs with supramaximal concentrations of ATP (1000 μM) or BzATP (30 μM) significantly prolonged recovery of current to baseline for wild-type $P2X_7$, but still failed to elicit a response for K193A or K311A mutants.

ATP and BzATP sensitivity of wild-type $P2X_7R$ expressed in *Xenopus* oocytes was determined from dose–response curves. Considerable care was taken to ensure adequate time had elapsed (20 min) between subsequent doses of agonists to ensure receptors were not desensitized. Data were only used where at least two responses to the same concentration of agonist produced identical currents as a measure of recovery from any desensitization.

Consistent with earlier work, sensitivity of wild-type $hP2X_7R$ to BzATP was significantly greater than to ATP [10,19,20] with EC_{50} of 46 μM and 23 μM and Hill coefficients of 1.96 and 2.14 ($n = 4$) for ATP and BzATP respectively (data not shown).

4. Discussion

Positively charged residues usually contribute to binding α

and γ phosphates of ATP, and these are usually lysine [21,22]. The present study shows removing the positive charge by substituting alanine for lysine at 193 and 311 abolished ATP-induced uptake of both small and large cation permeants.

These lysines are conserved throughout the P2X receptor family, and recent studies on $hP2X_1$ [4] and $rP2X_2$ [5] receptors suggest these lysines contribute to phosphate binding. Residues homologous to K311 in $P2X_1$ and $P2X_2$ exhibit total knockout of function when mutated in $P2X_7$. Mutating K193 to A in $P2X_7$ also results in total knockout of function, while in $P2X_2$ the effect is more muted and in $P2X_1$ the effect is further reduced, in line with an associated increase in ATP binding strength, with EC_{50}s of 100, 10 and 1 μM respectively [23]. Moreover, the observed effect on channel function correlates with the pore-forming phenotype of the three different receptor subtypes after continued exposure to agonist. $P2X_7$ forms pores much more capably than $P2X_2$, while $P2X_1$ appears unable to form pores.

Pro210 is conserved in mouse, rat and human $P2X_7$ sequences. Studies on the P210A mutant using fluorometry and flow cytometry indicated a partial reduction in receptor function compared with wild-type. P210 lies between K193, which we have shown is involved in an ATP binding site, and position 214. Replacement of the isoleucine at residue 214 is known to affect dissociation of agonist binding [9] and therefore affects the potency of ATP, indicating 214 lies in an ATP binding site. The P210A mutant attenuates $hP2X_7$ function, but is not imperative for binding of ATP. It is possible that agonist potency in the P210A mutant is affected through an increase in ATP dissociation resulting in a shorter pore opening time as evidenced by reduced ethidium uptake.

We found no evidence that truncated $hP2X_7$ (1–415) expressed on the surface of HEK293 cells since the extracellular epitopes were not accessible to our extracellular antibody (Fig. 1g,h). This suggests the C-terminal tail of $hP2X_7R$ is essential for trafficking the receptor to the cell surface, in contrast with $rP2X_7R$, the truncated form of which traffics to the cell surface and forms functional channels in HEK293 cells [10]. Truncated hP2X7R (1–439) expresses on the surface of *Xenopus* oocytes because currents are obtained [8]. This suggests segment 416–439 is essential for trafficking, or that there are significant differences in trafficking responses between amphibian and mammalian expression systems.

There have been few studies of the residues in the extracellular domain that are critical for agonist binding to $P2X_7$ or the stoichiometry of this binding. We have shown that P210A attenuates but does not abolish full function of the receptor, and that lysines K193 and K311 in $hP2X_7R$ are essential for ATP-induced channel and pore influx; whether they bind ATP phosphates in one site, or contribute to two different ATP binding sites within an oligomeric $hP2X_7R$, remains to be determined.

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