

Extracellular ATP Increases Cation Fluxes in Human Erythrocytes by Activation of the P2X₇ Receptor*

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Canine erythrocytes are known to undergo a reversible increase in cation permeability when incubated with extracellular ATP. We have examined the expression and function of P2X receptors on human erythrocytes using confocal microscopy and a panel of anti-P2X₁₋₇ antibodies and have measured monovalent cation fluxes in the presence of various nucleotide agonists. Human erythrocytes expressed P2X₇ receptors on all cells examined from eight of eight subjects, as well as P2X₂ at a far lower staining intensity in six of eight subjects. ATP stimulated the efflux of ⁸⁶Rb⁺ (K⁺) from human erythrocytes in a dose-dependent fashion with an EC₅₀ of ~95 μM. Other nucleotides also induced an efflux of ⁸⁶Rb⁺ from erythrocytes with an order of agonist potency of 2'- and 3'-O(4-benzoylbenzoyl) ATP (BzATP) > ATP > 2-methylthio-ATP (2MeSATP) > adenosine 5'-O-(3-thio-triphosphate) (ATPγS), whereas ADP or UTP had no effect. ATP-induced efflux of ⁸⁶Rb⁺ from erythrocytes was inhibited by extracellular Na⁺ and oxidized ATP, as well as by KN-62, an antagonist specific for the human P2X₇ receptor. When erythrocytes were incubated in isotonic KCl medium, the addition of ATP stimulated an ⁸⁶Rb⁺ influx approximately equal in magnitude to ATP-stimulated ⁸⁶Rb⁺ efflux from the same cells. BzATP also stimulated the influx of ²²Na⁺ into erythrocytes incubated in isotonic NaCl medium. Both ATP-induced efflux and influx of ⁸⁶Rb⁺ and ²²Na⁺ were impaired in erythrocytes from subjects who had inherited loss-of-function polymorphisms in the P2X₇ receptor. These results suggest that the reversible permeabilization of erythrocytes by extracellular ATP is mediated by the P2X₇ receptor.

concentration (1, 2), whereas the first description of Na⁺-K⁺-2Cl⁻ cotransport owed much to a nystatin technique to alter the Na⁺ gradient and change the driving force for cotransport (3). Yet another technique was introduced by Parker *et al.* (4, 5) who showed that intracellular Na⁺ and K⁺ concentration of canine erythrocytes could be equilibrated with the cation concentration in the medium simply by incubation with extracellular ATP for 30–60 min and that removal of ATP restored the basal permeability of the cell. This reversible effect of ATP suggested the involvement of an ATP-gated cation channel, but it is only with recent observations and knowledge of the P2X receptor family (6) that it is possible to study this question.

Seven subtypes of the P2X receptor family have been identified based on a common structure of two transmembrane domains with intracellular amino and carboxyl termini and a trimeric structure in the plasma membrane (6). Although P2X₁–P2X₆ channels all show desensitization in the continued presence of agonist (6), the converse occurs with the P2X₇ channel, which undergoes slow dilatation over 10–30 s to a second and larger permeability state allowing a massive loss of cellular K⁺ and a Na⁺ gain (7–9). A range of downstream events follow P2X₇ activation such as membrane blebbing (10) and microvesicle shedding (11) with the former requiring activation of Rho and p38 mitogen-activated protein kinases and an intact cytoskeleton (12–14). P2X₇ activation also leads to externalization of phosphatidylserine (11, 15) as well as the stimulation of the caspase cascade and a stress-activated protein kinase-dependent pathway resulting in apoptosis (16, 17). P2X₇ is variably expressed on the surface of leukocytes being absent on neutrophils, with moderate expression on lymphocytes and the highest expression on monocytes, dendritic cells, and macrophages (18–20).

We have previously described a loss-of-function polymorphism at nucleotide position 1513 of the human P2X₇ gene (1513A → C) that changes a glutamic acid to alanine at amino acid 496 (E496A) (21) located within an ankyrin-like repeat (22) or a tumor necrosis factor receptor 1-related death domain (23). Subjects who are homozygous for this polymorphism have grossly impaired ATP-induced Ca²⁺ and ethidium⁺ influx (21), Rb⁺ efflux (24), as well as ATP-induced cell death (21, 25, 26). The E496A mutation however does not impair ATP-induced currents in either *Xenopus* oocytes or HEK293 cells transfected with E496A mutant P2X₇ constructs (27), suggesting that this mutation although not affecting the initial opening of the channel somehow prevents its subsequent dilatation to a pore. More recently, we have identified another but less common loss-of-function single nucleotide polymorphism at nucleotide position 946 of the human P2X₇ gene (946G → A) that changes an arginine to glutamine at amino acid 307 (R307Q) (28). This residue is located within the ATP-binding pocket, which also includes other positively charged residues (Lys³¹¹ and Lys¹⁹³)

Many of our concepts of cellular Na⁺ and K⁺ homeostasis were based on experiments in the erythrocyte, a cell type in which intracellular Na⁺ and K⁺ concentrations could be readily changed and the subsequent effect on ion transport could be measured. Early studies of the sodium pump used the technique of hypotonic hemolysis and resealing of ghosts to study the dependence of Na⁺ pumping on intracellular cation

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that contribute to the binding of the negatively charged phosphate groups of ATP (29). The R307Q polymorphism in heterozygous dosage impairs ATP-induced Ca²⁺ and ethidium⁺ influx and Rb⁺ efflux, and in combination with the E496A polymorphism, also in heterozygous dosage, causes a complete loss of these fluxes (28). Using immunocytochemical, pharmacological and genetic approaches we have examined whether P2X₇ receptors are present on human erythrocytes. Our results showed that functional P2X₇ receptors are present on human erythrocytes although at lower abundance than on human lymphocytes.

EXPERIMENTAL PROCEDURES

Reagents—Bovine serum albumin (BSA),¹ D-glucose, glycerol gelatin mounting medium, saponin, ATP, BzATP, ADP, UTP, 2MeSATP, ATP_γS, OxATP, Me₂SO, ouabain, furosemide, and Drabkin's reagent (used according to the manufacturer's instructions) were from Sigma. KN-62 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). HEPES and normal horse serum were from Invitrogen. Rubidium-86 (⁸⁶Rb⁺) and sodium-22 (²²Na⁺) isotopes were obtained from PerkinElmer Life Sciences and Amersham Biosciences, respectively. Di-*n*-butyl phthalate and di-isooctyl phthalate (BDH Chemicals, Poole, England) were blended 80:20 (v/v) to give an oil mixture of density 1.03 g/ml. Rabbit polyclonal antibodies produced against extracellular epitopes of P2X₁₋₇ have been described (30, 31). The sheep polyclonal antibody produced against an extracellular epitope of P2X₇ has also been described (32). Cy2-conjugated donkey anti-rabbit or Cy3-conjugated donkey anti-sheep IgG antibodies were from Jackson Immuno-Research (West Grove, PA).

Erythrocytes—The study was approved by the Wentworth Area Health Service Human Ethics Committee (Penrith, Australia). Peripheral blood was collected in heparin-containing vacutainer tubes from 13 normal volunteers of various genotypes previously identified as either wild-type, heterozygous, or homozygous at nucleotide position 1513 (E496A) of the P2X₇ gene (21, 33). All subjects except one were wild-type at nucleotide positions 946 (Arg³⁰⁷) and 1729 (Ile⁵⁶⁸) of the P2X₇ gene (28, 32). One subject was a double heterozygote for both nucleotide 946A and 1513C (28). Blood was centrifuged at 400 × *g* for 15 min, and the plasma, platelets, leukocytes, and the upper 10% of erythrocytes were removed. The remaining erythrocytes were washed twice in NaCl medium (147.5 mM NaCl, 2.5 mM KCl, 5 mM D-glucose, 0.1% BSA, 20 mM HEPES, pH 7.5) at 1800 × *g* for 5 min.

Immunofluorescence Staining and Confocal Microscopy—Erythrocytes or whole blood cells were resuspended in NaCl medium at 5 × 10⁷ cells/ml, and 20 μl was smeared onto glass slides, air dried for 30 min, and fixed with acetone/methanol for 2 min (34). The slides were washed four times over 10 min with phosphate-buffered saline (PBS) and blocked with PBS, 20% normal horse serum, 0.1% BSA for 20 min before incubation for 45 min with anti-P2X antibody diluted in PBS, 0.2% normal horse serum. The slides were washed three times over 30 min with PBS and incubated for 45 min with fluorochrome-conjugated secondary antibody diluted in PBS, 0.2% normal horse serum. The slides were again washed before embedding in glycerol gelatin mounting medium. Cells were visualized with a Leica (Mannheim, Germany) TCS NT UV Laser Confocal Microscope as described (35).

⁸⁶Rb⁺ Efflux Measurements—Erythrocytes from wild-type subjects (unless as otherwise indicated) were loaded with ⁸⁶Rb⁺ (5 μCi/ml) at a hematocrit of 40% in NaCl medium for 4 h at 37 °C. Cells were then washed three times at 4 °C with ice-cold NaCl medium and resuspended in either KCl medium (150 mM KCl, 5 mM D-glucose, 0.1% BSA, 20 mM HEPES, pH 7.5) or NaCl medium at a final hematocrit of 5%. ⁸⁶Rb⁺-loaded erythrocyte suspensions (2.5 ml) were incubated in the absence or presence of nucleotide for 60 min at 37 °C. At 0- and 60-min time points, 1-ml samples were overlaid on 0.3 ml of phthalate oil mixture and centrifuged at 8000 × *g* for 30 s. For time-course studies, ⁸⁶Rb⁺-loaded erythrocyte suspensions (10 ml) were incubated in the absence or presence of 1 mM ATP for 4 h, with 1-ml samples collected at 30 min intervals as above. For studies using the P2X₇ antagonist, OxATP (36), ⁸⁶Rb⁺-loaded erythrocytes resuspended in NaCl medium

were pre-incubated in the absence or presence of 300 μM OxATP for 60 min at 37 °C, washed once with NaCl medium, and resuspended in KCl medium before incubation in the absence or presence of ATP for 60 min. For studies using the specific P2X₇ antagonist, KN-62 (37), ⁸⁶Rb⁺-loaded erythrocytes resuspended in KCl medium were pre-incubated in the presence of 1 μM KN-62 or an equal volume of Me₂SO vehicle for 15 min at 37 °C before incubation in the absence or presence of ATP for 60 min. Samples (700 μl) from the supernatant above the oil after centrifugation and a second sample (350 μl of ⁸⁶Rb⁺-loaded erythrocytes and 350 μl of 0.4% saponin) were collected to measure the level of radioactivity using a Wallac (Turku, Finland) 1480 Wizard 3 Automatic Gamma Counter. Hemolysis after a 60-min incubation was less than 1% as determined spectrophotometrically on cell-free supernatants with Drabkin's reagent (38).

⁸⁶Rb⁺ and ²²Na⁺ Influx Measurements—Erythrocyte suspensions (2 ml) at a final hematocrit of 5% in KCl or NaCl medium containing 1 mM furosemide and 100 μM ouabain (but no BSA) were incubated with ⁸⁶Rb⁺ or ²²Na⁺ (2 μCi/ml) in the absence or presence of 1 mM ATP or 0.2 mM BzATP, respectively, for up to 60 min at 37 °C. At 15, 30, 45, or 60 min, cells were washed three times with 10 ml of ice-cold isotonic saline (1200 × *g* for 60 s at 4 °C). Erythrocytes were lysed with 1 ml of 10 M NH₄OH and 100 μl of 1% saponin, the level of radioactivity was measured by γ counting, and the hemoglobin concentration was determined spectrophotometrically with Drabkin's reagent. Samples at each time point were performed in duplicate.

Statistical Analyses—The differences in ⁸⁶Rb⁺ release were compared using either the unpaired Student's *t* test for single comparisons to control samples (see Figs. 2 and 4) or analysis of variance for multiple comparisons (see Figs. 5 and 6 and Table I) using SPSS 11.5 for Windows (SPSS Inc, Chicago, IL) with *p* < 0.05 considered significant.

RESULTS

P2X₇ Receptors Are Present on Human Erythrocytes—We have previously detected P2X receptors on paraformaldehyde-fixed human lymphocytes using a panel of polyclonal antibodies and confocal microscopy (30); however, attempts to fix human erythrocytes immobilized onto poly(L-lysine)-coated coverslips or air-dried onto glass slides with paraformaldehyde were unsuccessful because of a large amount of cell loss (results not shown). Therefore, human erythrocytes were air-dried onto glass slides and fixed with acetone/methanol (34) and stained with a panel of polyclonal antibodies against all seven P2X subtypes. Confocal microscopy with two different anti-P2X₇ polyclonal antibodies (30, 32) showed P2X₇ to be present at low to moderate levels on all erythrocytes from eight of eight subjects tested (Fig. 1, A and B; results not shown). Of these subjects, five were wild-type (Fig. 1A), two were heterozygous (results not shown), and one was homozygous (Fig. 1B) for the E496A polymorphism. Labeling was diffuse, although occasional puncta were observed on erythrocytes from some subjects. P2X₂ was also present on erythrocytes; however, the intensity level of staining was much lower than that of P2X₇ (Fig. 1C) and was near to absent on erythrocytes from two of the eight individuals studied (results not shown). P2X₁, P2X₃, P2X₄, P2X₅, and P2X₆ were not detected, because the labeling using polyclonal antibodies to these subtypes was similar to that of pre-immune sera (Fig. 1D; results not shown). To determine the relative level of P2X₇ on erythrocytes compared with leukocytes, whole blood from two wild-type subjects was air-dried onto glass slides, fixed with acetone/methanol, and stained with anti-P2X₇ polyclonal antibodies. Confocal microscopy demonstrated two populations of stained cells within the whole blood smears, which morphologically resembled either erythrocytes or leukocytes, with the intensity level of staining much lower on erythrocytes than that of the mixed leukocyte population (Fig. 1, E and F).

P2X₇ Agonists Induce ⁸⁶Rb⁺ Efflux from Human Erythrocytes—The effect of extracellular ATP on cation fluxes in human erythrocytes was then studied. Cells were loaded with ⁸⁶Rb⁺ (a surrogate of K⁺) and incubated at 37 °C with 1 mM ATP for up to 4 h with samples collected at 30-min intervals. At

¹ The abbreviations used are: BSA, bovine serum albumin; ATP_γS, adenosine 5'-O-(3-thiotriphosphate); BzATP, 2'- and 3'-O(4-benzoyl-benzoyl) ATP; 2MeSATP, 2-methylthio-ATP; OxATP, oxidized ATP; PBS, phosphate-buffered saline; ⁸⁶Rb⁺, rubidium-86 isotope; ²²Na⁺, sodium-22 isotope.

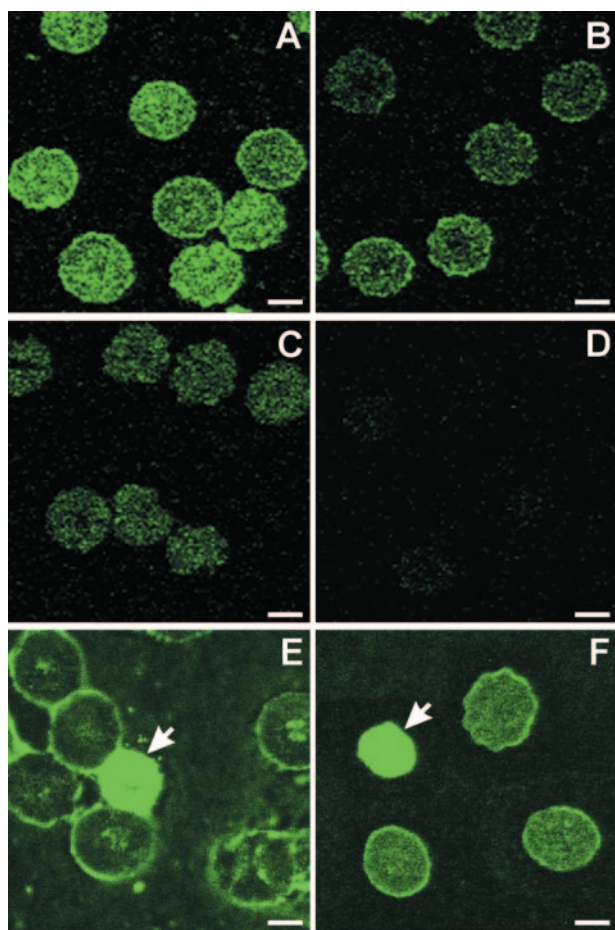


FIG. 1. Expression of P2X receptors on human erythrocytes. Erythrocytes (A–D) or whole blood cells (E and F) from subjects either wild-type at 496 (A and C–F) or homozygous for the E496A polymorphism (B) were incubated with rabbit polyclonal antibodies against P2X₇ (A, B, E, and F), P2X₂ (C), or P2X₁ (D) and subsequently with Cy2-conjugated anti-rabbit IgG antibody before examination by confocal microscopy. Arrows (E and F) indicate leukocytes (from two wild-type subjects) with higher levels of P2X₇ than adjacent erythrocytes. The expression of P2X₇ on erythrocytes and whole blood cells using a sheep anti-P2X₇ polyclonal antibody was similar to that observed using a rabbit anti-P2X₇ polyclonal antibody (results not shown). Pre-immune serum was routinely included as a negative control and demonstrated background labeling (results not shown) similar to that of P2X₁ (D) and P2X_{3–6} (results not shown). The calibration bar is 5 μm .

all time points, ATP induced a significant increment in ⁸⁶Rb⁺ efflux from erythrocytes compared with cells incubated in the absence of ATP (Fig. 2). The ATP-induced ⁸⁶Rb⁺ efflux was linear up to 60 min, therefore this time point was selected for all subsequent efflux studies.

The dose effect of ATP on cation fluxes in human erythrocytes was then studied. Cells loaded with ⁸⁶Rb⁺ were incubated at 37 °C with varying concentrations of ATP for 60 min. The efflux of ⁸⁶Rb⁺ from erythrocytes after a 60-min incubation ranged from 4.0 \pm 0.3% in the absence of a nucleotide up to 9.3 \pm 0.5% with 2 mM ATP (Fig. 3). Over this range of ATP concentrations an EC₅₀ value of 95.4 \pm 7.1 μM was obtained from the dose-response curve (Fig. 3), which is similar to values observed for either recombinant or native P2X₇ (EC₅₀ = 85 or 86 μM , respectively) (8, 39) but greater than that for P2X₂ (EC₅₀ = 1 μM) (40).

The effect of various nucleotide agonists on ⁸⁶Rb⁺ effluxes was then studied. Similar to above (Fig. 2), the release of ⁸⁶Rb⁺ from erythrocytes after a 60-min incubation in the absence of nucleotide was 3.6 \pm 0.4% (Fig. 4). The P2X₇ agonists, 0.2 mM BzATP and 1 mM ATP, induced a significant efflux of ⁸⁶Rb⁺

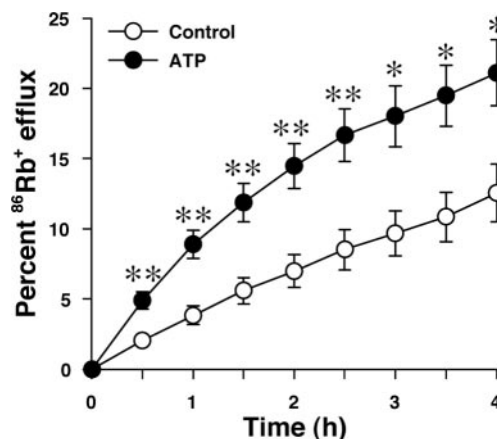


FIG. 2. Time course for ATP-induced ⁸⁶Rb⁺ efflux from human erythrocytes. ⁸⁶Rb⁺-loaded erythrocytes resuspended in KCl medium were incubated at 37 °C for up to 4 h in the absence (○) or presence (●) of 1 mM ATP. ⁸⁶Rb⁺ efflux was calculated as the difference in percentage release between 0 min and the times indicated. The release of ⁸⁶Rb⁺ from erythrocytes at 0 min in the absence and presence of ATP was 1.0 \pm 0.1 and 1.1 \pm 0.1%, respectively, and was not significantly different ($p = 0.43$). Results are expressed as the mean \pm S.E. from four experiments. *, $p < 0.02$ or **, $p < 0.01$ to control sample at corresponding time point.

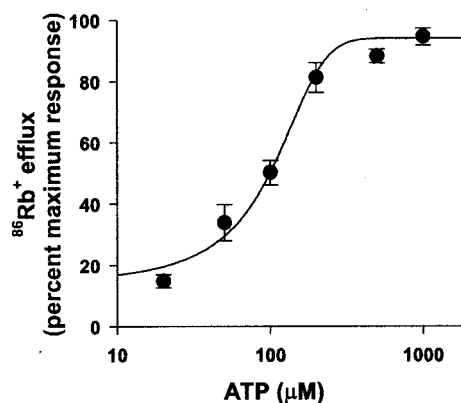


FIG. 3. Dose-response curve for ATP-induced ⁸⁶Rb⁺ efflux from human erythrocytes. ⁸⁶Rb⁺-loaded erythrocytes resuspended in KCl medium were incubated at 37 °C for 60 min in the presence of ATP (as indicated). ⁸⁶Rb⁺ efflux, calculated as the difference in percentage release between 0 and 60 min, was used to determine the percentage of maximal response to 2 mM ATP. Results are expressed as the mean \pm S.E. from five experiments.

from erythrocytes incubated in KCl medium with 12.0 \pm 1.2 and 9.9 \pm 0.9% of the total cell-associated ⁸⁶Rb⁺ being released at 60 min, respectively (Fig. 4). Other P2X₇ agonists, 1 mM 2MeSATP and 1 mM ATP γ S, which are both partial agonists (39), also induced ⁸⁶Rb⁺ efflux (6.8 \pm 0.3 and 4.7 \pm 0.6%, respectively), although the latter failed to reach significance compared with ⁸⁶Rb⁺ efflux in the absence of the nucleotide ($p = 0.15$). Thus for human erythrocyte P2X₇, the agonist potencies follow a rank order of BzATP > ATP > 2MeSATP > ATP γ S, which is identical to the order found for recombinant and native P2X₇ (8, 39). ADP and UTP, added at either 1 mM (Fig. 4) or 0.1 mM (results not shown) gave a ⁸⁶Rb⁺ release similar to control values, thus excluding a role for many of the P2Y receptors some of which are on avian erythrocytes (41).

Na⁺ Impairs ATP-induced ⁸⁶Rb⁺ Efflux from Human Erythrocytes—A characteristic of the P2X₇ receptor is its sensitivity to inhibition by extracellular Na⁺ (42, 43). Therefore, nucleotide-induced ⁸⁶Rb⁺ release from erythrocytes incubated in either KCl or NaCl medium was compared. ⁸⁶Rb⁺ efflux in response to either 1 or 0.1 mM ATP was significantly lower in NaCl

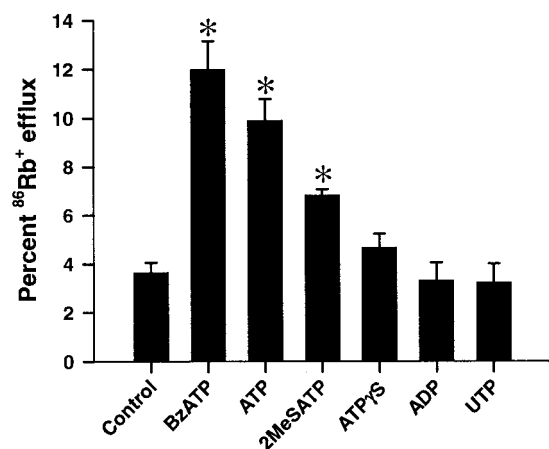


FIG. 4. P2X₇ agonists induce ⁸⁶Rb⁺ efflux from human erythrocytes. ⁸⁶Rb⁺-loaded erythrocytes resuspended in KCl medium were incubated at 37 °C for 60 min in the absence (Control) or presence of 1 mM nucleotide (as indicated) except BzATP (0.2 mM). ⁸⁶Rb⁺ efflux was calculated as the difference in percentage release between 0 and 60 min. Results are expressed as the mean ± S.E. from 3–6 experiments. *, *p* < 0.001 to control sample.

medium compared with KCl medium (6.0 ± 0.8 versus 10.8 ± 1.8% and 4.1 ± 0.5 versus 7.4 ± 1.1%, respectively; Fig. 5). The release of ⁸⁶Rb⁺ from erythrocytes suspended in NaCl or KCl media in the absence of nucleotide was identical (3.2 ± 0.7 and 3.2 ± 0.8%, respectively; Fig. 5).

P2X₇ Antagonists Inhibit ATP-induced ⁸⁶Rb⁺ Efflux from Human Erythrocytes—To confirm that ATP-induced ⁸⁶Rb⁺ efflux from human erythrocytes was mediated by P2X₇ activation, ⁸⁶Rb⁺-loaded cells were pre-incubated in the absence or presence of the P2X₇ antagonists, OxATP (36) or KN-62 (37), and the ATP-induced ⁸⁶Rb⁺ effluxes measured. Incubation with 300 μM OxATP completely inhibited both 1 mM and 0.1 mM ATP-induced ⁸⁶Rb⁺ efflux (Table I). Similarly, the incubation of cells with 1 μM KN-62 significantly inhibited both 1 and 0.1 mM ATP-induced ⁸⁶Rb⁺ efflux by over 90% (Table I). Neither OxATP nor KN-62 altered the basal release of ⁸⁶Rb⁺ from erythrocytes incubated in the absence of ATP (Table I).

Cells with a Loss-of-Function Polymorphism in the P2X₇ Receptor Have Absent ATP-induced Fluxes—We assessed whether BzATP or ATP could stimulate the efflux of ⁸⁶Rb⁺ from erythrocytes of subjects who were homozygous for the loss-of-function polymorphism E496A and whose mononuclear leukocytes lack P2X₇ function (21). Incubation of erythrocytes from these polymorphic subjects with either 0.2 mM BzATP or 1 mM ATP produced a release of ⁸⁶Rb⁺ of 1.9 ± 0.0 and 1.9 ± 0.1%, respectively, which was similar to that released from either homozygote or wild-type erythrocytes in the absence of nucleotide (2.0 ± 0.2 and 3.3 ± 0.6%, respectively) (Fig. 6). In the same experiments, BzATP and ATP induced a significant release of ⁸⁶Rb⁺ from wild-type erythrocytes of 12.8 ± 1.4 and 9.6 ± 0.5%, respectively (Fig. 6). BzATP and ATP also failed to induce ⁸⁶Rb⁺ release from homozygote erythrocytes incubated in NaCl medium (results not shown).

P2X₇ Activation Increases K⁺ and Na⁺ Influx into Human Erythrocytes—The P2X₇ cation-selective channel/pore shows little or no selectivity between K⁺ and Na⁺ ions (6). We compared the ability of P2X₇ agonists to stimulate ⁸⁶Rb⁺ and ²²Na⁺ influx into erythrocytes from wild-type subjects. To reduce the flux of K⁺ and Na⁺ by other transporters, erythrocytes were incubated in the presence of ouabain, a Na⁺/K⁺ ATPase inhibitor (1), and furosemide, an inhibitor of the Na⁺-K⁺-2Cl⁻ cotransporter (3). ATP (1 mM) induced an influx of K⁺ into wild-type erythrocytes at a rate of ~4.0 μmole K⁺/ml of cells/h (Fig. 7A). This rate of influx is comparable with that of

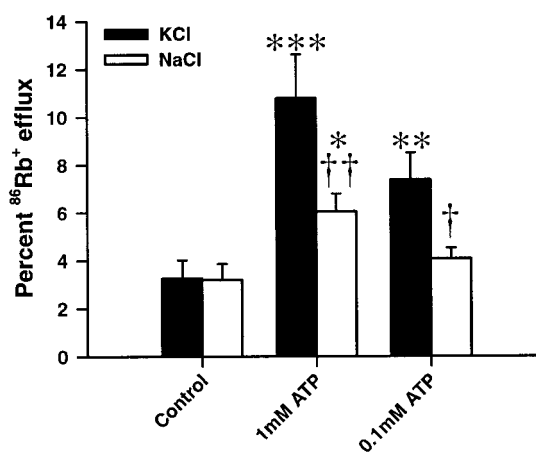


FIG. 5. Na⁺ impairs ATP-induced ⁸⁶Rb⁺ efflux from human erythrocytes. ⁸⁶Rb⁺-loaded erythrocytes resuspended in either KCl or NaCl media were incubated at 37 °C for 60 min in the absence (Control) or presence of ATP (as indicated). ⁸⁶Rb⁺ efflux was calculated as the difference in percentage release between 0 and 60 min. Results are expressed as the mean ± S.E. from three experiments. *, *p* < 0.05; **, *p* < 0.01 or ***, *p* < 0.001 to corresponding control sample; †, *p* < 0.05 or ††, *p* < 0.01 to corresponding ATP-treated sample in KCl medium.

TABLE I
P2X₇ antagonists inhibit ATP-induced ⁸⁶Rb⁺ efflux from human erythrocytes

⁸⁶Rb⁺-loaded erythrocytes were pre-incubated for 60 min in the absence (Control) or presence of 300 μM OxATP or for 15 min in the presence of Me₂SO vehicle or 1 μM KN-62, before incubation at 37 °C for 60 min in the absence (No ATP) or presence of ATP (as indicated) in KCl medium. ⁸⁶Rb⁺ efflux was calculated as the difference in percentage release between 0 and 60 min.

| Additions | Percent ⁸⁶ Rb ⁺ efflux ^a | | |
|--------------------|---|--------------------------|--------------------------|
| | No ATP | 1 mM ATP | 0.1 mM ATP |
| Control | 2.40 ± 0.85 | 8.33 ± 1.18 ^b | 5.74 ± 0.53 ^c |
| OxATP | 2.65 ± 0.86 | 2.65 ± 0.74 ^d | 2.54 ± 0.84 ^e |
| Me ₂ SO | 2.90 ± 1.11 | 8.07 ± 1.05 ^e | 6.41 ± 1.12 ^f |
| KN-62 | 3.07 ± 1.13 | 3.46 ± 1.12 ^e | 3.02 ± 1.46 ^g |

^a Results are expressed as the mean ± S.E. from three experiments.

^b *p* < 0.001 to a corresponding sample in the absence of ATP.

^c *p* < 0.01 to a corresponding sample in the absence of ATP.

^d *p* < 0.001 to a corresponding ATP-treated sample in the absence of antagonist.

^e *p* < 0.01 to a corresponding ATP-treated sample in the absence of antagonist.

^f *p* < 0.05 to a corresponding sample in the absence of ATP.

^g *p* < 0.05 to a corresponding ATP-treated sample in the absence of antagonist.

ATP-induced K⁺ efflux, which we estimate to be ~6.0 μmole K⁺/ml cells/h, assuming a net ATP-induced ⁸⁶Rb⁺ efflux of ~6.7% over 60 min (Fig. 4) and an intracellular K⁺ concentration of ~90 μmole/ml of cells. BzATP (0.2 mM) also induced an influx of Na⁺ into wild-type erythrocytes at a similar rate (~5.2 μmole Na⁺/ml of cells/h; Fig. 7B). In contrast, ATP and BzATP failed to induce an influx of K⁺ (~0 μmole K⁺/ml of cells/h) and Na⁺ (~0.2 μmole Na⁺/ml of cells/h), respectively, into erythrocytes from a subject who was a double heterozygote for two loss-of-function polymorphisms in P2X₇ at amino acid positions 307 and 496 (28) (Fig. 7).

DISCUSSION

Over three decades ago, Parker and Snow (4) demonstrated that extracellular ATP could reversibly increase the permeability of canine erythrocytes to both K⁺ and Na⁺ ions. This effect of ATP was specific to this nucleotide, because neither ADP nor UTP increased cation fluxes and, moreover, the permeability increase could be impaired by the addition of Mg²⁺, suggesting a role for the ATP⁴⁻ species. Here we report a similar phenom-

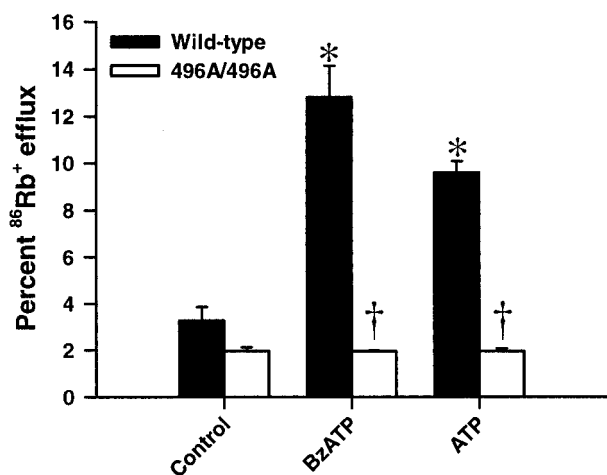


FIG. 6. BzATP- and ATP-induced ⁸⁶Rb⁺ efflux is impaired from human erythrocytes homozygous for the E496A polymorphism. ⁸⁶Rb⁺-loaded erythrocytes from subjects either wild-type at 496 (closed bars) or homozygous for the E496A polymorphism (open bars) were resuspended in KCl medium and incubated at 37 °C for 60 min in the absence (Control) or presence of 0.2 mM BzATP or 1 mM ATP. ⁸⁶Rb⁺ efflux was calculated as the difference in percentage release between 0 and 60 min. Results are expressed as the mean ± S.E. from four experiments. *, *p* < 0.001 to corresponding control sample; †, *p* < 0.001 to corresponding nucleotide-treated wild-type sample.

enon for human erythrocytes and show that P2X₇ mediates this effect of extracellular ATP. We developed two different anti-P2X₇ polyclonal antibodies (30, 32), and using immunolabeling and confocal microscopy we showed P2X₇ expression on human erythrocytes. Both polyclonal antibodies recognize non-homologous epitopes in the extracellular domain of the P2X₇ receptor (residues 71–86 and 65–81), and both have been previously shown to label P2X₇-transfected but not mock-transfected HEK293 cells (29, 32). These antibodies can also detect native P2X₇ receptors on human lymphocytes (30), and on the basis of fluorescence intensity we estimate that P2X₇ is at least 10-fold less abundant on human erythrocytes than on lymphocytes (Fig. 1). Similarly, measurements of cation permeability of human erythrocytes by standard isotope flux techniques showed that the magnitude of the ATP-stimulated K⁺ and Na⁺ fluxes are far less for human erythrocytes than for lymphocytes. Many features of the erythrocyte fluxes closely resemble those resulting from activation of the P2X₇ receptor either as the native molecule in mononuclear leukocytes or expressed heterologously in HEK293 cells. First, the EC₅₀ for ATP (~95 μM) and the rank order of agonist potency (BzATP > ATP > 2MeSATP > ATP_γS) were typical of recombinant and native P2X₇ (8, 39). Second, ATP-induced ⁸⁶Rb⁺ efflux was lower from erythrocytes incubated in NaCl medium compared with KCl medium, and it is well documented that Na⁺ inhibits the activation of P2X₇ (42, 43). Third, ATP-induced ⁸⁶Rb⁺ efflux was inhibited by two known P2X₇ antagonists, OxATP (36) and the more specific inhibitor, KN-62 (37). Fourth, ATP and BzATP produced approximately the same increase in the influx of ⁸⁶Rb⁺ and ²²Na⁺, respectively, into erythrocytes, consistent with P2X₇ being a channel/pore showing little or no selectivity between these two monovalent cations (6). Finally, ATP and BzATP-induced fluxes were absent in erythrocytes of three subjects, two being homozygous for the E496A loss-of-function polymorphism (21) and the third being a double heterozygote for the R307Q and E496A loss-of-function polymorphisms (28).

Activation of native P2X₇ by extracellular ATP produces a nearly complete release of ⁸⁶Rb⁺ from human lymphocytes within 5 min (42, 44) and from monocytes within 2 min (24), as well as from murine macrophages (45). In contrast, the ATP-

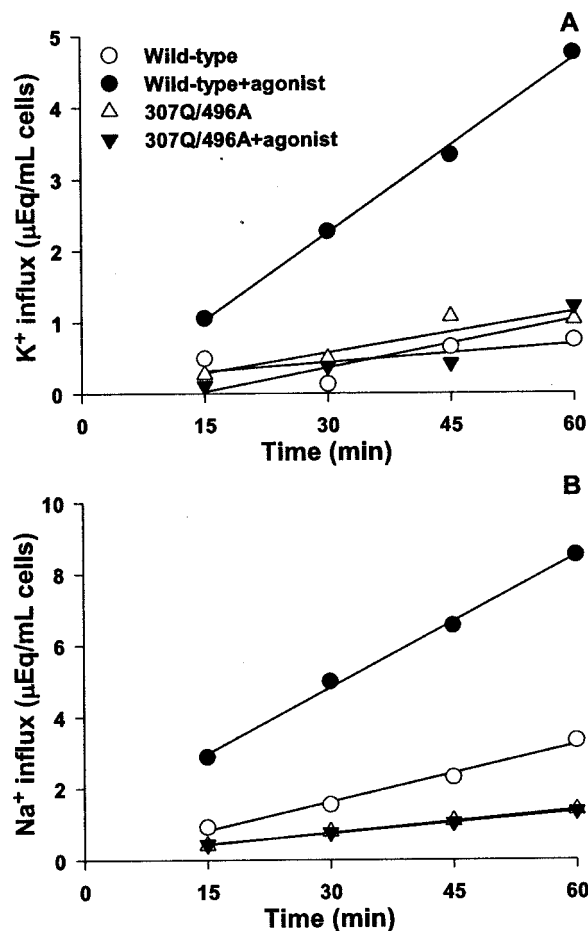


FIG. 7. P2X₇ agonists induce K⁺ and Na⁺ influx into human erythrocytes. Erythrocytes from subjects either wild-type at 307 and 496 (○, ●) or heterozygous for both R307Q and E496A polymorphisms (△, ▼) were resuspended in either KCl medium (containing ⁸⁶Rb⁺, 1 mM furosemide and 100 μM ouabain but no BSA) (A) and incubated at 37 °C in the absence (○, △) or presence (●, ▼) of 1 mM ATP or NaCl medium (containing ²²Na⁺, 1 mM furosemide and 100 μM ouabain but no BSA) (B) and incubated at 37 °C in the absence (○, △) or presence (●, ▼) of 0.2 mM BzATP. ⁸⁶Rb⁺ and ²²Na⁺ uptake was calculated as the μeq of K⁺ or Na⁺/ml of erythrocytes, respectively. Results are expressed as the mean from duplicate time points.

induced ⁸⁶Rb⁺ efflux from human erythrocytes was much slower with 9.5 ± 0.3% (*n* = 19) of the cell-associated ⁸⁶Rb⁺ being released after a 60-min incubation with 1 mM ATP. We have shown previously that the level of P2X₇ expression on lymphocytes and monocytes correlates with the level of ATP-induced permeabilization to ethidium⁺ (18), suggesting that the slow rate of ATP-induced ⁸⁶Rb⁺ release from erythrocytes is because of the low level of P2X₇ expression on these cells. In addition, the observation that only ~10% of the cell-associated ⁸⁶Rb⁺ is released after a 60-min incubation with ATP and that beyond this time point the release is not linear with time (Fig. 2) suggests that ATP induces the release of ⁸⁶Rb⁺ from a subpopulation of erythrocytes or that the ⁸⁶Rb⁺ is released from an intracellular compartment within these cells. It is of interest that ATP-induced cation fluxes in human erythrocytes are much lower than those in their canine counterparts (4, 5, 46). In canine erythrocytes, 0.5 mM ATP induced a near to total release of ⁴²K⁺ after a 40-min incubation (4) suggesting a K⁺ efflux at least one order of magnitude greater than shown in Fig. 2 for human erythrocytes. Sha'afi and colleagues (46) also demonstrated that 1 mM ATP could induce a 7-fold increase in Na⁺ influx into canine erythrocytes resuspended in a phos-

phate-buffered NaCl medium but had little effect on either human or feline erythrocytes. Thus like other mechanisms of cation transport in mammalian erythrocytes (47), those mediated by extracellular ATP also differ between species.

Fig. 6 shows that the ATP-induced ⁸⁶Rb⁺ efflux was almost absent from erythrocytes of subjects homozygous for the E496A polymorphism. This polymorphism impairs ATP-induced ethidium⁺ influx and ⁸⁶Rb⁺ efflux through the dilated pore in leukocytes (21, 24) but does not impair ATP-induced currents that are mediated via the P2X₇ channel in either *Xenopus* oocytes or HEK293 cells transfected with E496A mutant P2X₇ constructs (27). Our results suggested that the ATP-induced ⁸⁶Rb⁺ efflux occurs via the second and larger permeability state (dilated pore) of the P2X₇ receptor and that isotope fluxes of ⁸⁶Rb⁺ through the smaller P2X₇ channel are too small to be reliably measured in this cell type. Moreover, our data showed that ATP-stimulated ⁸⁶Rb⁺ efflux and influx are approximately equal for erythrocytes suspended in 150 mM KCl, and both are similar to the BzATP-stimulated Na⁺ influx for cells suspended in a similar concentration of NaCl (147.5 mM). Therefore, these results exclude a role for an ATP-gated rectifier channel in the ATP-induced flux of cations in human erythrocytes.

In addition to P2X₇, we also detected P2X₂ receptors on human erythrocytes using immunocytochemistry and confocal microscopy. However, despite the P2X₂ channel being permeable to Rb⁺ ions (48), three results suggest that P2X₂ receptors contributed little to the ATP-induced ⁸⁶Rb⁺ efflux. First, P2X₂ expression on erythrocytes was lower than P2X₇, possibly by as much as one order of magnitude, although we can not rule out that the difference in expression between these two purinoreceptors may be because of differences in the immunoreactivity of the antibodies used. Second, erythrocytes that lacked P2X₂, as determined by confocal microscopy, displayed ATP-induced ⁸⁶Rb⁺ efflux. Finally, as discussed above, the rank order of agonist potency (BzATP > ATP > 2MeSATP > ATP γ S) and the EC₅₀ for ATP (95.4 \pm 7.1 μ M) are typical of P2X₇, whereas for human P2X₂, the four nucleotides BzATP, ATP, 2MeSATP and ATP γ S are equipotent with an EC₅₀ of \sim 1 μ M (40). The failure to detect any functional P2X₂ may simply reflect the low level of expression of this receptor on human erythrocytes. In one study, extracellular ATP stimulated a regulatory volume decrease, which reversed the hypotonic swelling of *Necturus* erythrocytes via activation of a receptor characteristic of P2X₂ (49, 50); however, a similar mechanism could not be observed in human erythrocytes (49). Nonetheless, differences in P2X expression between erythrocytes of different species are likely to be found.

It is likely that P2X₇ has functional significance for human erythrocytes, because it is well established that activation of P2X₇ causes apoptotic death of a variety of cell types (51). Recent studies have shown that an influx of Ca²⁺ carried by Ca²⁺ ionophores leads to phosphatidylserine externalization and the apoptosis of erythrocytes (52, 53). Hypertonic shock and oxidative stress also increase phosphatidylserine externalization and erythrocyte apoptosis, and both stressors have been shown to open Ca²⁺-permeable channels in the membrane (53, 54). These channels have many of the features of P2X₇, because both are permeable to Ca²⁺ and monovalent cations, and both are partly inhibited by high concentrations (1 mM) of amiloride (42, 53, 54). Moreover, the externalization of phosphatidylserine on erythrocytes via P2X₇ activation may lead to their subsequent phagocytosis by macrophages. Such a mechanism has been demonstrated for human erythrocytes exposed to either Ca²⁺ ionophores or oxidative stress (52, 55). In support of a role for P2X₇ in the apoptosis of erythrocytes, preliminary

experiments indicate that BzATP can induce phosphatidylserine externalization on these cells.² Therefore, the expression of functional P2X₇ receptors on the surface of erythrocytes suggests that this receptor has a role in the apoptotic death of this cell after its 120-day life span.

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