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$P2X_7$ receptor activation causes phosphatidylserine exposure in human erythrocytes

Ronald Sluyter *, Anne N. Shemon, James S. Wiley

Department of Medicine, Nepean Clinical School, University of Sydney, Penrith, NSW, Australia

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Abstract

Activation of cation channels causes erythrocyte phosphatidylserine (PS) exposure and cell shrinkage. Human erythrocytes express the $P2X_7$ receptor, an ATP-gated cation channel. The two most potent $P2X_7$ agonists, BzATP and ATP, stimulated PS exposure in human erythrocytes. Other nucleotides also induced erythrocyte PS exposure with an order of agonist potency of BzATP > ATP > 2MeSATP > ATPγS; however neither ADP nor UTP had an effect. ATP induced PS exposure in erythrocytes in a dose-dependent fashion with an EC_{50} of ~75 μM. BzATP- and ATP-induced erythrocyte PS exposure was impaired by oxidised ATP, as well as in erythrocytes from subjects who had inherited loss-of-function polymorphisms in the $P2X_7$ receptor. ATP-induced PS exposure in erythrocytes was not significantly altered in the presence of EGTA excluding a role for extracellular Ca^{2+}. These results show that $P2X_7$ activation by extracellular ATP can induce PS exposure in erythrocytes.

Keywords: $P2X_7$; P2Z; Purinergic receptor; Red blood cell; Phosphatidylserine

The asymmetrical distribution of phospholipid in normal erythrocytes is well maintained with phosphatidylserine (PS) residing exclusively in the inner leaflet of the membrane [1]. Treatment of erythrocytes with Ca^{2+} ionophores, hypertonic shock, energy depletion or oxidative stress induces PS exposure and cell shrinkage [2–4]. Both these events are due to activation of Ca^{2+}-permeable cation channels causing a net increase in cytosolic Ca^{2+} [4] and subsequent activation of the Ca^{2+}-sensitive Gardos channel leading to K^+ loss [5].

The $P2X_7$ receptor is a ligand-gated cation channel which upon activation by extracellular ATP causes a loss of cellular K^+ and gain of Ca^{2+} and Na^+ [6]. A range of downstream events follow $P2X_7$ activation in leukocytes including PS exposure [7,8]. Using immunocytochemistry and confocal microscopy, as well as measurements of cation fluxes we have shown that human erythrocytes express functional $P2X_7$ receptors [9]. Whether signalling events downstream of $P2X_7$ activation occur in erythrocytes however remains unknown. Therefore, we investigated if activation of the $P2X_7$ receptor on human erythrocytes could induce PS exposure.

Materials and methods

Materials. ATP, 2′- and 3′-0(4-benzoylbenzoyl) ATP (BzATP), 2-methylthio-ATP (2MeSATP), adenosine 5′-0-(3-thiotriphosphate) (ATPγS), ADP, UTP, ATP-2′,3′-dialdehyde (OxATP), and Drabkin's Reagent were from Sigma (St. Louis, MO). Annexin-V-FLUOS was from Roche Diagnostics (Pensberg, Germany).

Erythrocytes. The study was approved by the Sydney West Area Health Service Human Ethics Committee. Erythrocytes were isolated from peripheral blood from eight volunteers as previously described [9]. Six subjects were wild-type at nucleotide positions 151 + 1, 946, 1096, 1513, and 1729 of the *P2RX7* gene [10], while two subjects were either homozygous for the 1513C polymorphism or compound heterozygous for the 151 + 1t and 1513C polymorphisms [11].

PS exposure assay. Erythrocytes were resuspended in NaCl medium (147.5 mM NaCl, 2.5 mM KCl, 5 mM D-glucose, 20 mM Hepes, pH 7.5) at a final concentration of 2% and added to 96-well U-bottom plates.

* Corresponding author. Fax: +61 4734 3432.
E-mail address: rons@med.usyd.edu.au (R. Sluyter).

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NaCl medium (control) or nucleotide (prepared in NaCl medium) was added to the erythrocytes and incubated at 37 °C and 95% air/5% CO_2 for 24 h. The ATP^{4-} concentration in solution was calculated using the Bound and Determined Program [12].

After 24-h incubation, erythrocytes were washed in Annexin-binding buffer (NaCl medium containing 5 mM $CaCl_2$) and labelled with Annexin-V-FLUOS according to the manufacturer's instructions. Annexin-V binding (PS exposure) and forward scatter (cell size) was measured by flow cytometry (FACSCalibur, Becton–Dickinson, San Jose, CA). The percentage of hemolysis was determined by hemoglobin measurements of supernatants and cell lysates assayed spectrophotometrically with Drabkin's Reagent according to the manufacturer's instructions.

Statistics. Differences were compared using either the unpaired Student's *t*-test for single comparisons or ANOVA for multiple comparisons with $P < 0.05$ considered significant.

Results

We have previously demonstrated that human erythrocytes express functional $P2X_7$ receptors [9]. Since $P2X_7$ activation is known to cause PS exposure in leukocytes [7,8], we investigated if the two most potent $P2X_7$ agonists, BzATP and ATP, could induce erythrocyte PS exposure as measured by Annexin-V binding. Incubation of erythrocytes from six different subjects with either 200 μM BzATP or 1 mM ATP for 24 h induced significant PS exposure compared to erythrocytes incubated in the absence of nucleotide ($P < 0.01$; Fig. 1). BzATP- and ATP-induced PS exposure however occurred in the absence of cell shrinkage as cell size did not significantly alter between the three treatments (results not shown). Similarly BzATP and ATP also failed to induce haemolysis, with less than 2% of haemoglobin being released from erythrocytes after 24-h incubation and no significant differences between treatments (results not shown).

The effect of other nucleotide agonists on erythrocyte PS exposure was then studied. As above, 200 μM BzATP and 1 mM ATP induced significant erythrocyte PS exposure compared to control values (Fig. 2A). The partial $P2X_7$ agonists, 1 mM 2MeSATP and ATPγS also increased PS exposure on erythrocytes although the latter failed to reach significance ($P = 0.11$). Agonists of P2Y receptors and other P2X receptors, 1 mM ADP and UTP, failed to increase PS exposure compared to control values.

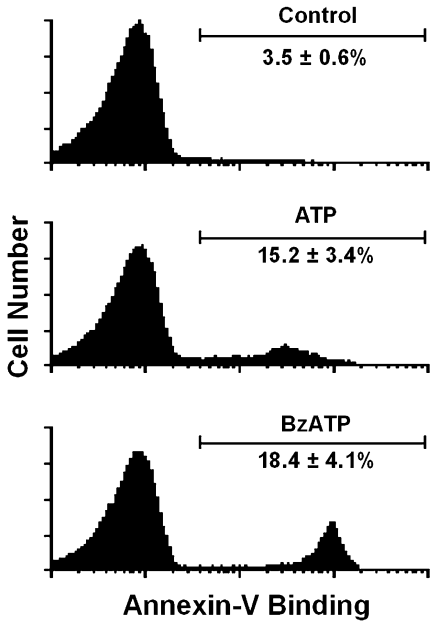

Fig. 1. BzATP and ATP induce erythrocyte PS exposure. Erythrocytes were incubated in the absence (control) or presence of 1 mM ATP or 200 μM BzATP at 37 °C for 24 h. Erythrocytes were labelled with Annexin-V-FLUOS and the level of Annexin-V binding measured. Marker regions (as shown) were used to calculate the percentage of Annexin-V binding. Mean percentages ± SD ($n = 6$) are shown; representative histograms from one subject are shown.

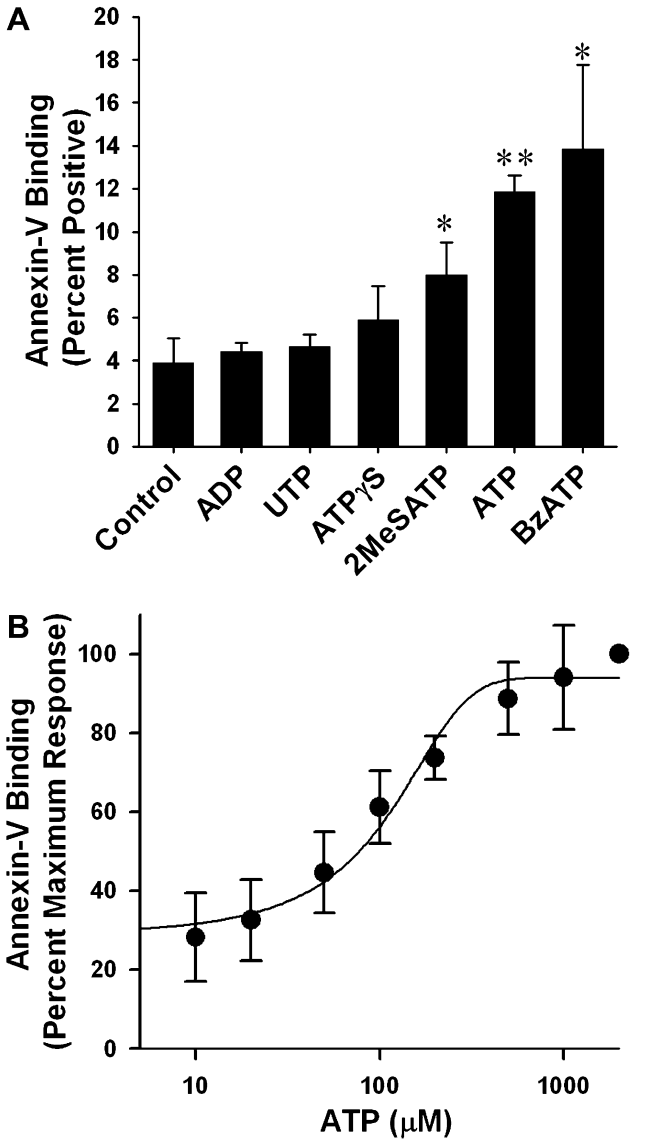

Fig. 2. $P2X_7$ agonists induce erythrocyte PS exposure. Erythrocytes were incubated in the (A) absence (control) or presence of 1 mM nucleotide or 200 μM BzATP or (B) varying ATP concentrations at 37 °C for 24 h. Erythrocytes were labelled with Annexin-V-FLUOS and the level of Annexin-V binding measured. (A) Results are expressed as the mean ± SD ($n = 3$–4); $^{*}P < 0.05$ or $^{**}P < 0.01$ to control. (B) Results are expressed as the mean ± SD ($n = 4$).

The dose effect of ATP on erythrocyte PS exposure was then studied. Annexin-V binding on erythrocytes after 24-h incubation ranged from 4.1 ± 1.0% in the absence of nucleotide up to 12.1 ± 1.3% with 2 mM ATP, and yielded an EC_{50} value of 75 ± 33 μM (Fig. 2B).

To confirm that BzATP- and ATP-induced PS exposure in human erythrocytes was mediated by $P2X_7$ activation, erythrocytes were pre-incubated in the absence or presence of the $P2X_7$ antagonist, OxATP [13], and nucleotide-induced PS exposure measured. Pre-incubation of erythrocytes with 300 μM OxATP inhibited 200 μM BzATP- and 1 mM ATP-induced PS exposure by 68.1 ± 16.4% and 88.8 ± 13.8%, respectively (Fig. 3A).

We next assessed whether BzATP or ATP could stimulate PS exposure in erythrocytes from subjects who carried loss-of-function polymorphisms on both $P2X_7$ alleles and whose leukocytes lack $P2X_7$ function [11]. Incubation of polymorphic erythrocytes with either 200 μM BzATP or 1 mM ATP failed to induce significant PS exposure, with Annexin-V binding significantly lower than that induced by either agonist on wild-type erythrocytes (Fig. 3B). Annexin-V binding on polymorphic and wild-type erythrocytes incubated in the absence of nucleotide was similar.

Previous studies by others have shown that PS exposure on erythrocytes due to Ca^{2+} ionophores, hypertonic shock, energy depletion or oxidative stress is dependent on an influx of extracellular Ca^{2+} [3–5]. In our system, however

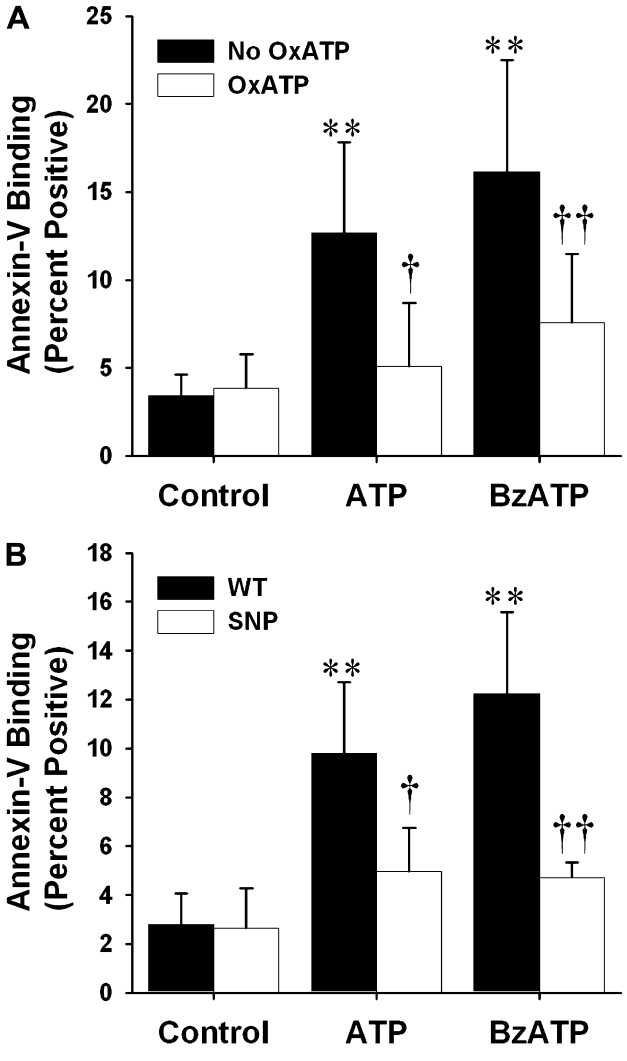

Fig. 3. BzATP and ATP induce PS exposure on erythrocytes via $P2X_7$ activation. (A) Erythrocytes, pre-incubated for 60 min in the absence or presence of 300 μM OxATP, or (B) erythrocytes, from wild-type or loss-of-function polymorphic subjects, were incubated in the absence (control) or presence of 1 mM ATP or 200 μM BzATP at 37 °C for 24 h. Erythrocytes were labelled with Annexin-V-FLUOS and the level of Annexin-V binding measured. Results are expressed as the mean ± SD ($n = 4$); $^{**}P < 0.01$ to corresponding control (A and B); $^{\dagger}P < 0.05$ or $^{\dagger\dagger}P < 0.01$ to (A) corresponding nucleotide-treated sample in the absence of OxATP or (B) corresponding wild-type sample.

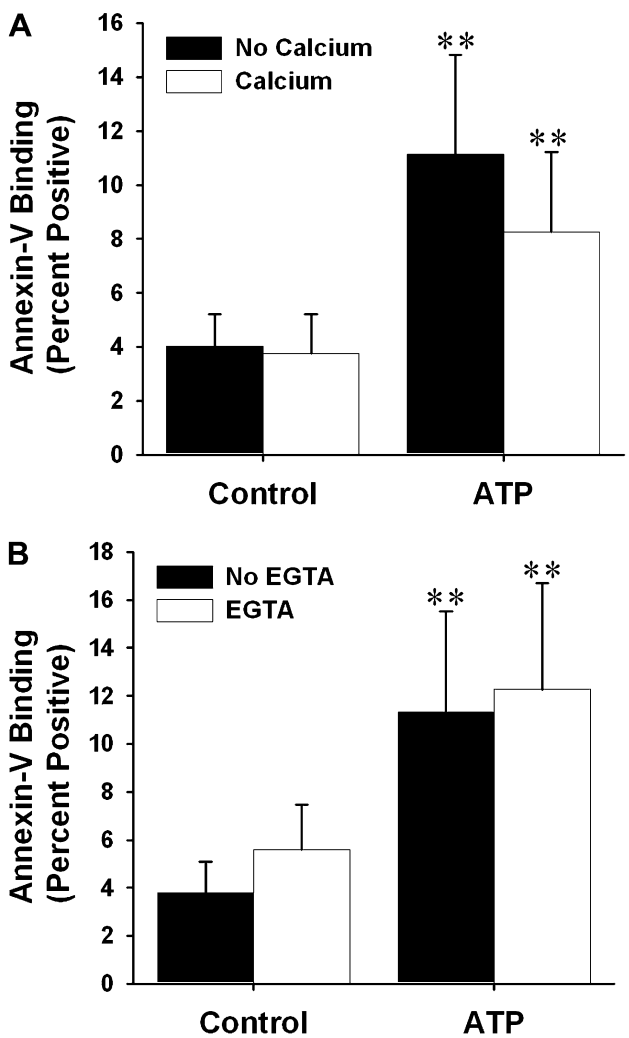

Fig. 4. Extracellular Ca^{+} is not required for ATP-induced erythrocyte PS exposure. Erythrocytes (A) in the absence or presence of 1 mM Ca^{2+} were incubated in the absence (control) or presence of 1 mM ATP without Ca^{2+} (251 μM ATP^{4-}) or 1.54 mM ATP with Ca^{2+} (251 μM ATP^{4-}) or (B) in the absence or presence of 100 μM EGTA were incubated in the absence (control) or presence of 1 mM ATP at 37 °C for 24 h. Erythrocytes were labelled with Annexin-V-FLUOS and the level of Annexin-V binding measured. Results are expressed as the mean ± SD ($n = 8$); $^{**}P < 0.01$ to corresponding control.

BzATP and ATP induce erythrocyte PS exposure in NaCl medium nominally free of Ca^{2+}. Therefore, we investigated if 1 mM Ca^{2+}, as used by others [3–5], could potentiate the ATP-induced erythrocyte PS exposure. Cells were incubated in the absence or presence of 1 mM Ca^{2+} and ATP. Since free Ca^{2+} however lowers the concentration of ATP^{4-}, the form responsible for $P2X_7$ activation [6], cells in the presence or absence of 1 mM Ca^{2+} were incubated with 1.54 or 1 mM ATP, respectively, to provide equimolar ATP^{4-} concentrations (251 μM). PS exposure on erythrocytes incubated with ATP in the presence or absence of additional Ca^{2+} was similar and significantly higher than PS exposure on erythrocytes incubated in the absence of ATP (Fig. 4A). Since additional Ca^{2+} did not alter ATP-induced PS exposure, we examined whether extracellular Ca^{2+} was required for this process in erythrocytes. Cells were incubated in the presence or absence of 1 mM ATP in NaCl medium containing 100 μM or no EGTA, a Ca^{2+} chelator. ATP induced a significant and similar level of PS exposure on erythrocytes incubated in the presence or absence of EGTA (Fig. 4B). Annexin-V binding on erythrocytes incubated in the presence or absence of EGTA but without ATP was similar.

Discussion

In this current study, we demonstrate that extracellular BzATP and ATP can induce erythrocyte PS exposure. This process was mediated via activation of the $P2X_7$ receptor despite the presence of other P2 receptors on human erythrocytes, such as $P2X_2$ [9], $P2Y_1$ [14], and $P2Y_{13}$ [15]. First, the rank order of agonist potency (BzATP > ATP > 2MeSATP > ATPγS) is typical of cation fluxes mediated by recombinant $P2X_7$ [6] and erythrocyte $P2X_7$ [9]. While the inability of the $P2Y_1$ agonist, UTP and the $P2X_2$ and $P2Y_{13}$ agonist, ADP at either 1 mM or 100 μM (results not shown) to induce erythrocyte PS exposure exclude a role for these P2 receptors in this process. Second, the EC_{50} for ATP-induced erythrocyte PS exposure of ~75 μM is comparable to that of cation fluxes mediated by recombinant $P2X_7$ [6] and erythrocyte $P2X_7$ [9]. Third, BzATP- and ATP-induced erythrocyte PS exposure were inhibited by the $P2X_7$ antagonist, OxATP [13]. Fourth, BzATP- and ATP-induced PS exposure were markedly reduced in erythrocytes from subjects who carried loss-of-function polymorphisms on both $P2X_7$ alleles [11].

PS exposure in human erythrocytes by activation of $P2X_7$ differs to that of other known treatments [2–4] in that PS exposure occurs in the absence of extracellular Ca^{2+}. A possible explanation for this difference is that $P2X_7$-induced erythrocyte PS exposure is a result of Na^+ influx. Activation of erythrocyte $P2X_7$ is known to induce an influx of Na^+ [9], while $P2X_7$ activation on murine thymocytes is known to induce rapid PS exposure on the cell surface, a process dependent on Na^+ influx and which can occur in the absence of extracellular Ca^{2+} [7]. Increases in extracellular Na^+ may directly activate the phospholipid transporter and inhibit the aminophospholipid translocase thus causing PS externalisation but preventing PS internalization, respectively [7]. Alternatively the influx of Na^+ may deplete intracellular ATP through its consumption by the Na^+/K^+ pump as a result of removing Na^+ from the cells [7]. In support of this, exposure of canine erythrocytes to extracellular ATP leads to decreased intracellular ATP levels [16]. Regardless of the exact mechanism, $P2X_7$ activation can mediate PS exposure on erythrocytes without any involvement of the Gardos channel.

PS exposure in human erythrocytes by $P2X_7$ activation also differed to other models in that PS exposure occurred in the absence of cell shrinkage as measured by forward scatter, a flow cytometric parameter of cell size. Lang and colleagues [17] have demonstrated detectable erythrocyte shrinkage by measuring the forward scatter of cells, following exposure to Pb^+, which can cause a level of Annexin-V binding similar to $P2X_7$-induced PS erythrocyte exposure. Thus it appears unlikely that our measurements of cell size by flow cytometry lacked sufficient sensitivity to detect changes in cell volume.

$P2X_7$ activation and subsequent PS exposure may play a role in the destruction of erythrocytes after their 120 day life-span or in vascular dysfunction. PS exposure on erythrocytes can lead to recognition and removal by splenic macrophages [1]. Thus erythrocytes exposing PS as a result of $P2X_7$ activation may be recognised and removed by splenic macrophages and thus removed from the circulation. Alternatively, erythrocyte $P2X_7$ may play a role in splenic "conditioning". During circulation, erythrocytes are often detained for many hours in the slow transient compartment of the spleen in close apposition to macrophages. Splenic "conditioning" of erythrocytes leads to loss of membrane material and a progressive increase in osmotic fragility of the red cell population [18], whether PS exposure on erythrocytes is a cause or a result of "conditioning" is uncertain, but our data suggest that the role of erythrocyte $P2X_7$ should be considered in this process. Whatever the role, our data demonstrate that $P2X_7$ activation can lead to the gain or loss of cations [9], as well as downstream signalling events such as PS exposure in erythrocytes.

Acknowledgments

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