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RESEARCH PAPER

Stimulation of rat erythrocyte $P2X_7$ receptor induces the release of epoxyeicosatrienoic acids

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Background and purpose: Red blood cells (RBCs) are reservoirs of vasodilatory, antiaggregatory, and antiinflammatory lipid mediators—epoxyeicosatrienoic acids (EETs). This study addresses the formation and release of erythrocyte-derived EETs in response to ATP receptor stimulation that may represent an important mechanism regarding circulatory regulation.
Experimental approach: Erythrocyte EET formation and release were investigated by incubating rat RBCs in physiological salt solution with agents that effected ATP release via P2 receptor stimulation of phospholipase A2 and epoxygenase-like activities with activation of the ATP secretory mechanism. EETs were analyzed by gas and liquid chromatography-mass spectrometry.
Key results: EETs were released from rat RBCs: 14,15-, 11,12-, 8,9- and 5,6-EETs in a ratio of 1.2:1.0:0.9:0.8. EETs were produced by epoxidation of arachidonic acid catalyzed by hemoglobin. Spontaneous release of EETs, 0.66 ± 0.14 ng per 10^9 RBCs, was dose-dependently increased by an ATP analog, BzATP, and inhibited by $P2X_7$ receptor antagonists. 5 μM ATP increased release of EETs over 20% to 0.83 ± 0.15 ng per 10^9 RBCs; 10 μM BzATP tripled the amount of EET release to 1.87 ± 0.20 ng per 10^9 RBCs. EET release by ATP or BzATP was not associated with hemolysis. Carbenoxolone, a gap junction inhibitor that inhibits ATP release, and glibenclamide, an inhibitor of the cystic fibrosis transmembrane conductance regulator (CFTR), which is required for ATP release, inhibited the spontaneous and stimulated EET release from RBCs.
Conclusions and implications: EETs are produced and released from RBCs via a mechanism that is mediated by ATP stimulation of $P2X_7$ receptors coupled to ATP transporters, pannexin-1 and CFTR.
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Keywords: ATP; purinoceptors; erythrocytes; haemoglobin; epoxyeicosatrienoic acids; CFTR; gap junction; pannexin-1; microcirculation

Abbreviations: ABC, ATP-binding cassette; ATPγ*S*, adenosine 5′-*O*-(3-thiotriphosphate); BBG, brilliant blue G; BEL, bromoenol lactone; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; CFTR, cystic fibrosis transmembrane conductance regulator; EET, epoxyeicosatrienoic acid; ESI LC-MS, electrospray ionization liquid chromatography-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; HEPES, *N*-2-hydroxyethylpiprazine-*N*′-2-ethanesulphonic acid; HETE, hydroxyeicosatetraenoic acid; MAFP, methyl arachidonyl flurophosphonate; 2MeSATP, 2-methylthio-ATP; MSPPOH, methylsulphonyl propargyloxyphenyl hexanamide; ODYA, 17-octadecenoic acid; PLA_2, phospholipase A2; PSS, physiological salt solution; RBC, red blood cell

Introduction

We have reported that red blood cells (RBCs) are reservoirs for *cis*- and *trans*-epoxyeicosatrienoic acids (EETs) (Jiang *et al*., 2004, 2005). EETs are essential components of key vasoregulatory mechanisms and are candidate endothelium-derived hyperpolarizing factors (Quilley *et al*., 1997; Spector *et al*., 2004). As EETs mediate pleiotropic biological responses, including those affecting ion transport (Pascual *et al*., 1998; Watanabe *et al*., 2003), gene expression (Node *et al*., 2001; Michaelis and Fleming, 2006) vasorelaxation (Imig *et al*., 2001; Carroll *et al*., 2006), inflammation (Node *et al*., 1999; Campbell, 2000; Liu *et al*., 2005) and platelet aggregation (Fitzpatrick *et al*., 1987; Jiang *et al*., 2004), the release of EETs from RBCs is replete with potential contributions to the control of the microcirculation and the rheological properties of the circulating blood.

RBCs can regulate microvascular tone, which has been explored via mediators ranging from ATP (Ellsworth *et al*., 1995; Dietrich *et al*., 2000), nitric oxide (NO) (Jia *et al*., 1996; Stamler *et al*., 1997; McMahon *et al*., 2002) and haemoglobin (Jagger *et al*., 2001) to hypoxia-inducible factors (Hagen *et al*., 2003; Singel and Stamler, 2005). Through dynamic

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interactions between NO and haemoglobin, RBCs can elicit hypoxic vasodilation (Jia *et al*., 1996; McMahon *et al*., 2002). Coronary blood flow during exercise has been proposed to be regulated by ATP released from RBCs (Farias III *et al*., 2005). RBC deformation-induced ATP release requires the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) (Sprague *et al*., 1998). In addition, pannexin-1 was recently identified as an ATP release channel in RBCs (Locovei *et al*., 2006). However, the release of vasoactive lipid mediators from RBCs has not been reported.

More than three decades ago, Parker and Snow (1972) demonstrated that external ATP influenced the permeability and metabolism of dog erythrocytes. ATP acts as an extracellular signalling molecule via interactions with specific P2 receptors to mediate a wide variety of processes (Burnstock *et al*., 1972; Burnstock and Knight, 2004). Extracellular ATP increases cation fluxes by activating the $P2X_7$ receptor, which has been identified as the major form of P2X receptors in human erythrocytes (Sluyter *et al*., 2004). Activation of the $P2X_7$ receptor has been associated with cytoskeletal rearrangement, membrane internalization, pore formation and permeability and trafficking changes (Kim *et al*., 2001; Kochukov and Ritchie, 2004). In response to deformation of RBCs and hypoxaemia on passage through the microcirculation (Bergfeld and Forrester, 1992; Sprague *et al*., 1998), ATP release from RBCs may represent an important mechanism involved in the regulation of the microcirculation.

ATP has the additional capability of activating secretion of EETs from RBCs, an effect that should greatly amplify the vascular response to ATP. EET release from rat RBCs was analysed by gas chromatography-mass spectrometry (GC-MS) as well as electrospray ionization liquid chromatography-mass spectrometry (ESI LC-MS). We have shown that (1) RBCs metabolized arachidonic acid (AA) to form EETs; (2) stimulation of the erythrocyte $P2X_7$ receptors by ATP or an ATP analog, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP), increased EET release; (3) inhibition of phospholipase A2 (PLA_2) prevented EET release from RBCs; (4) inhibition of ATP release by blocking either transporters or channels, CFTR or pannexin-1, reduced EET release from RBCs and (5) EET release from RBCs was not associated with haemolysis.

Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. Eight-week-old male Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were maintained at 22°C with alternating cycles of light and darkness and fed *ad libitum* with standard rat chow and water.

RBC preparation

Sprague–Dawley rats (9–12-week old) were anaesthetized with pentobarbital, 65 mg kg^{-1} intraperitoneal and 10 ml blood was drawn from the inferior vena cava after midline laparotomy using heparin rinsed syringes and transferred to Vacuette heparin tubes (Fisher). After inverting 4–6 times, the blood was cooled on ice and then centrifuged at 800 *g* at 4°C for 10 min. The supernate and the buffy layer were removed by aspiration. Packed RBCs were washed three times and resuspended in a physiological salt solution (PSS, in mM: 4.0 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 15.7 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 11.1 dextrose, and 2 mg ml^{-1} bovine serum albumin, pH adjusted to 7.35). The RBCs were examined and counted under the microscope, and the RBC solution was diluted with PSS to 2×10^9 RBCs ml^{-1}.

RBC incubation and lipid extraction

RBCs (1–2 ml per sample) were incubated with or without treatment in a VWR incubating mini shaker at 37°C for 30 min with shaking around a 3 mm orbit at 600 r.p.m. Agents in the experiments were prepared in stock solutions at 100 to 200-fold of the targeted concentration and 1% of the solvents, including ethanol and dimethyl sulphoxide, were tested without significant effects. After incubation, the mixture was centrifuged at 2000 *g* for 10 min. Internal standards, EET-d8 (6 ng) and AA-d8 (10 ng), were added to aliquots of the incubation buffer and extracted with 2 ml ethyl acetate two times after adjusting pH to 4 with acetic acid. The combined organic phase was dried, reconstituted in acetonitrile for high-performance liquid chromatography (HPLC) separation and ESI LC-MS analysis. HPLC was carried out using Shimadzu LC-10AT Liquid Chromatograph with automatic sample injection and programmed fraction collection. A Beckman-Coulter Ultrasphere ODS column (25 cm × 4.6 mm × 5 μm) was used. Ultraviolet (UV) absorbance from 200 to 400 nm was monitored. A gradient from 75% acetonitrile–25% water–0.05% acetic acid to 100% acetonitrile in 20 min was used. Total EETs were collected from 11.8 to 14.8 min and AA was collected from 19.5 to 20.6 min.

Haemolysis measurements

The RBC incubation buffer, after centrifugation at 2000 *g* for 10 min, was diluted 1:4 with phosphate-buffered saline and the UV absorbances at 414 nm were measured with the subtraction of the average baselines at 380 and 450 nm (Malinauskas, 1997). The haemoglobin concentration was calculated from a standard curve using rat haemoglobin. Freeze–thawing of RBCs was carried out by freezing RBCs at −70°C for 10 min and warming up at 37°C. Haemoglobin of the freeze–thawed samples after centrifuging the ghosts at 2000 *g* for 20 min was measured by diluting 220-fold in 0.01% Na_2CO_3 and calculated according to Harboe (1959) method.

Quantitation of AA and total EETs

For quantitation of AA and total EETs using negative ion chemical ionization GC-MS, AA and EETs were derivatized to pentafluorobenzyl esters (Jiang *et al*., 2004). The GC column (DB-1 ms, 10 m length, 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies Inc.) was temperature programmed from 150 to 250°C at a rate of 30°C min^{-1} for AA and EET detection. Methane was used as the reagent gas. The ions of *m/z* 303 and 311 for AA/AA-d8 and *m/z* 319 and 327 for EET/EETd8 were monitored. The total amounts of AA and EETs were calculated from their peak area ratios according to standard curves.

ESI LC-MS analyses

ESI LC-MS analyses of EETs were carried out as described (Jiang *et al*., 2005). Briefly, a Finnigan LCQ Advantage quadrupole ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with ESI source run by XCALIBUR software was used. HPLC was run with a Luna C18(2) 250 × 2.0 mm column (Phenomenex, Torrance, CA, USA) with an isocratic gradient of acetonitrile–water–methanol–acetic acid (60:30:10:0.05) at a flow rate of 0.25 ml min^{-1}. ESI was carried out at an ion transfer tube temperature of 260°C, a spray voltage of 4.5 kV, a sheath gas flow of 34 units and an auxiliary gas flow of 20 units (units refer to arbitrary values set by the LCQ software).

Data analyses

Data were presented as mean ± s.e. of *n* experiments. An unpaired Student's *t*-test and one-way analysis of variance were performed to test for differences between two groups. A value of $P<0.05$ was regarded as statistically significant.

Reagents

All chemicals including AA, ATP, BzATP, 2-methylthio-ATP (2MeSATP), adenosine 5′-*O*-(3-thiotriphosphate) (ATPγ*S*), methyl arachidonyl flurophosphonate (MAFP), bromoenol lactone (BEL), S3319, HEPES, bovine serum albumin and rat haemoglobin were purchased from Sigma-Aldrich (St Louis, MO, USA). AA was used after further purification by HPLC. Methyl sulphonyl propargyloxyphenyl hexanamide (MSPPOH) and 17-octadecenoic acid (ODYA) were synthesized by Dr John R Falck. Acetonitrile, methanol and hexanes (all HPLC grade) were purchased from Fisher Scientific. EET-d8 standards were obtained from Biomol.

Results

EETs released from RBCs

Analysis by ESI LC-MS of the basal release of lipid mediators from RBCs revealed hydroxyeicosatetraenoic acids (HETEs) and EETs (Figure 1a). The identification of HETEs and EETs was based on their HPLC retention times and tandem mass spectra (Brash *et al*., 1995; Jiang *et al*., 2004). RBCs released spontaneously 13-, 10-, 15-, 7- and 12-HETEs as well as four EETs. The EETs released were 14,15-, 11,12-, 8,9- and 5,6-*cis*-EETs in a ratio of 1.2:1.0:0.9:0.8, both in control and in response to stimulation with ATP, BzATP and AA (Figures 1b–d).

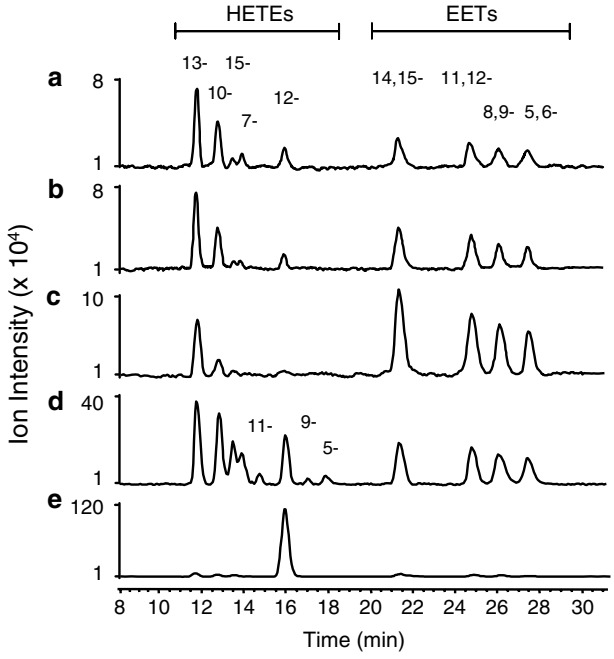

Figure 1 Representative ESI LC-MS profiles ($n=4$–8, *m/z* 319) of the released HETEs and EETs from incubations of control (**a**), ATP 1 mM (**b**), BzATP 10 μM (**c**), AA 0.66 μM (**d**) and A23187 2 μM (**e**) with 4×10^9 rat RBCs in 2 ml buffer for 30 min at 37°C. Eicosanoid products from RBCs include 15-, 13-, 12-, 11-, 10-, 9-, 7- and 5-HETEs as well as 14,15-, 11,12-, 8,9- and 5,6-EETs. EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid.

Both ATP at 1 mM and BzATP at 10 μM increased the release of EETs and, although less apparent for ATP, they inhibited the release of HETEs (Figures1b and c). Exposure of RBCs with 2 μM calcium ionophore A23187 selectively stimulated the release of 12-HETE (Figure 1e), as has been reported (Kobayashi and Levine, 1983).

Addition of 0.66 μM AA to 4×10^9 RBCs in 2 ml PSS resulted in the synthesis and release of all of the HETEs and EETs (Figure 1d). When reacting the solution from the lysed RBCs or from purified rat haemoglobin with AA, various product profiles including formation of all *cis*- and *trans*-EETs were observed depending on the ratio of AA and haemoglobin concentrations (data not shown). The formation of total EETs from AA (1 μg ml^{-1}) in the haemoglobin (1 mg ml^{-1}) solution from lysed RBCs at 37°C for 30 min was inhibited by both the cytochrome *P*450 epoxygenase inhibitor, MSPPOH and the combined inhibitor of both the ω-hydroxylases and epoxygenases, ODYA (Table 1). The same level of EET formation and inhibition was also found for AA when reacting with purified rat haemoglobin (Sigma, St Louis, MO, USA).

Extracellular ATP on EET release from RBCs

ATP from 1 μM to 1 mM produced a multiphasic response on EET release (Figure 2). A total of 1 mM ATP increased EET release over 40% relative to the spontaneous release

Table 1 Conversion of AA (1 μg ml^{-1}) to EETs by haemoglobin (1 mg ml^{-1}) from lysed rat erythrocytes and inhibition by MSPPOH or ODYA ($n=4$)

	Control	*MSPPOH (24 μM)*	*ODYA (30 μM)*
EET (ng μg^{-1} AA)	83.75±7.22	56.75±8.88*	68.45±3.35*

Abbreviations: AA, arachidonic acid; EET, epoxyeicosatrienoic acid; MSPPOH, methylsulphonylpropargyloxyphenyl hexanamide; ODYA, 17-octadecyenoic acid.
Data are expressed as mean±s.e.
*$P<0.05$, as compared with control.

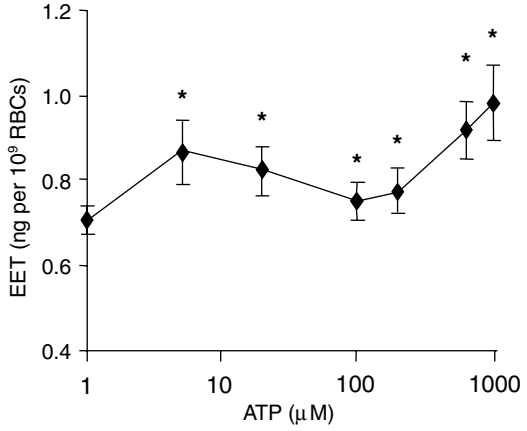

Figure 2 Total EET release from rat erythrocytes in response to extracellular ATP. ATP from 1 μM to 1 mM was incubated with rat erythrocytes for 30 min at 37°C. $n=8$–12, *$P<0.05$ vs spontaneous EET release at 0.66±0.14 ng per 10^9 RBCs. EET, epoxyeicosatrienoic acid.

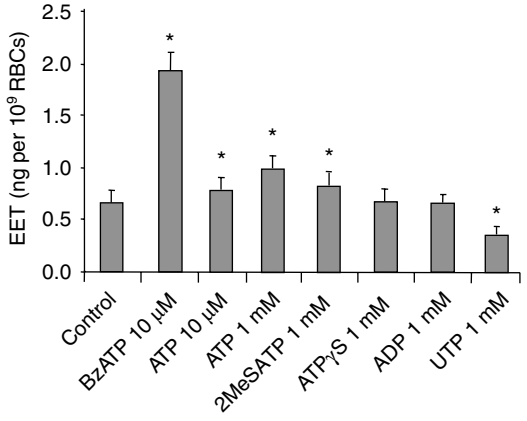

Figure 3 Comparison of the potency of P2 receptor agonists on total EET release from rat erythrocytes. Rat RBCs were incubated with ATP, BzATP, 2MeSATP, ATPγS, ADP and UTP for 30 min at 37°C. EETs were analysed by GC-MS from the supernatent after centrifuging the RBCs. $n=4$–8, *$P<0.05$ vs control. BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; EET, epoxyeicosatrienoic acid; 2MeSATP, 2-methylthio-ATP.

(Figure 3). ATP at 5 μM, a concentration found in human plasma (Gonzalez-Alonso *et al*., 2002), increased EET release over 20% (0.83±0.15 ng/10^9 RBC). Suramin (100 μM), considered to be a specific P2Y$_2$ receptor antagonist (Charlton *et al*., 1996), stimulated EET release (Table 2), while reactive blue-2 (RB-2, 50 μM), a non-specific ATP antagonist, inhibited EET release, suggesting a dual action of ATP on the EET releasing mechanisms in rat RBCs, which also explained the multiphasic effects of ATP on EET release from RBCs shown in Figure 2. Suramin, at 300 μM, inhibited EET release (data not shown) revealing non-specific P2 receptor inhibition at high concentrations.

Table 2 Effects of P2 antagonists on EET release from rat erythrocytes (2×10^9 cells ml^{-1}, $n=4$–8)

	Basal	*Suramin (100 μM)*	*RB-2 (50 μM)*
Control	0.66±0.14	0.97±0.15*	0.44±0.08*
ATP (1 mM)	0.98±0.16	1.49±0.28*	0.68±0.12*

Abbreviations: EET, epoxyeicosatrienoic acid; RB-2, reactive blue-2.
All values were expressed as ng per 10^9 RBCs.
Data are expressed as mean±s.e.
*$P<0.05$ as compared with basal.

P2X$_7$ receptor stimulation increased EET release from RBCs

Comparison of ATP with other P2 receptor agonists showed that stimulation of EET release was in the order of BzATP >2MeSATP >ATPγS (Figure 3), typical of the response of P2X$_7$ receptors (Surprenant *et al*., 1996; Gargett *et al*., 1997). BzATP was most potent in releasing EETs from RBCs, consistent with P2X$_7$ receptors serving as the predominant P2X receptor in human erythrocytes (Sluyter *et al*., 2004) and BzATP acting as a potent selective P2X$_7$ receptor agonist (Baraldi *et al*., 2004). UTP (1 mM), mostly a P2Y receptor agonist (Kumari *et al*., 2003), inhibited EET release from RBCs ($P<0.05$ vs basal; Figure 3), which as indicated, can also explain the multiphasic effect of ATP on EET release from RBCs.

BzATP dose-dependently stimulated EET release with an EC$_{50}$ of 6.6 μM (Figure 4). 100 μM BzATP more than tripled the spontaneous EET release. Although RBCs were routinely incubated with shaking at 37°C for 30 min after adding the reagents to the cold RBCs, the addition of 10 μM BzATP 2 min before finishing the incubation resulted in the same increase in EET release as produced by adding BzATP at the start of the 30 min incubation, indicating rapid formation and release of EETs by activating P2X$_7$ receptors in RBCs. P2X$_7$ antagonists, brilliant blue G (BBG, 10 μM) and Zn^{2+} (100 μM), inhibited ATP (1 mM) and BzATP (10 μM) stimulated EET release (Figure 5).

Effects of ATP release inhibitors on EET releases from RBCs

Both CFTR (Sprague *et al*., 1998) and pannexin-1 (Locovei *et al*., 2006) have been separately reported to mediate the release of ATP from RBCs although there are likely to be interactions between CFTR and pannexin (Kotsias and Peracchia, 2005). Glibenclamide and niflumic acid, inhibitors of CFTR activities, inhibited ATP release during erythrocyte deformation (Sprague *et al*., 1998). Carbenoxolone, an inhibitor of gap junctions, inhibited ATP release from RBCs induced by hypotonic stress (Locovei *et al*., 2006).

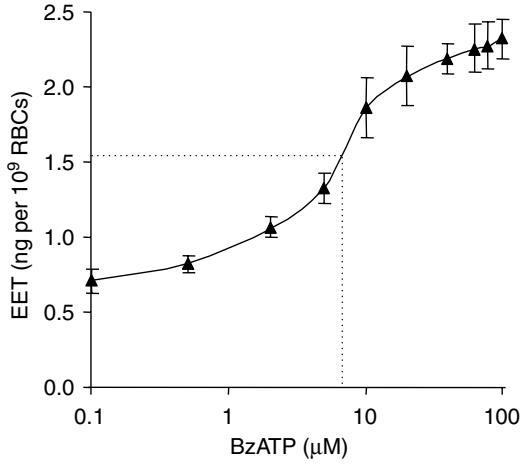

Figure 4 Dose-dependent response of the $P2X_7$ receptor agonist, BzATP, on EET release from rat erythrocytes. Incubations were carried out with BzATP from 0.1 to 100 μM. $n=$ 6–10. BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; EET, epoxyeicosatrienoic acid.

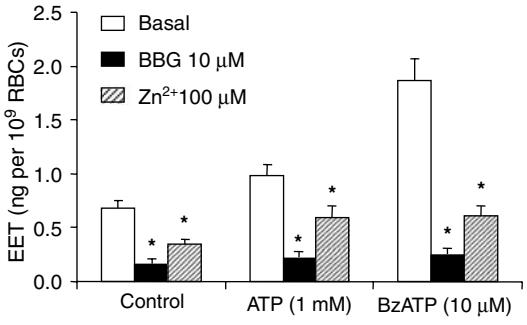

Figure 5 $P2X_7$ antagonists, BBG and Zn^{2+}, inhibit EET release from rat erythrocytes. $n=6$–10, $^*P<0.05$ vs basal in the same group. BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; BBG, brilliant blue G; EET, epoxyeicosatrienoic acid.

These inhibitors, all blocked spontaneous, as well as ATP and BzATP stimulated EET release (Figure 6).

Role of PLA_2 on EET release from RBCs

Effects of the inhibitors of Ca^{2+}-dependent and independent cytosolic PLA_2 (MAFP and BEL, respectively), and secretory PLA_2 (S3319) on EET release from RBCs were tested (Figure 7). MAFP at 10 μM most effectively inhibited EET release. To a much lesser degree, BEL and S3319 inhibited EET release from RBCs.

Haemolysis and AA release

Haemolysis and AA release were also monitored in the incubates to assess their effects on EET release from RBCs. Stimulated EET release by ATP and BzATP was not associated with increases in haemolysis or AA release into the buffer (Table 3). To induce haemolysis, a toxic concentration of $Cu(NO_3)_2$ (100 μM) was used in the incubation of RBCs. However, EET release was not increased by elevated haemolysis and AA release. Freezing and thawing of RBCs released lower amounts of EETs than basal EET release. The total AA in rat RBCs was analysed and found to be 18.07 ± 0.68 μg per 10^9 RBCs, making the spontaneous AA release from RBCs (Table 3) account for about 0.1% of the total AA in RBCs.

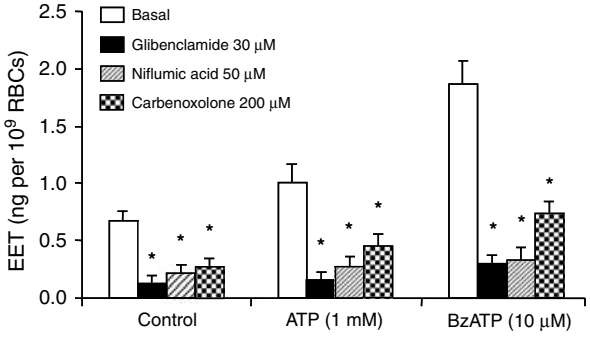

Figure 6 Effects of ATP release inhibitors, glibenclamide, niflumic acid and carbenoxolone, on EET release from rat erythrocytes. $n=6$–12, $^*P<0.05$ vs basal in the same group. BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; EET, epoxyeicosatrienoic acid.

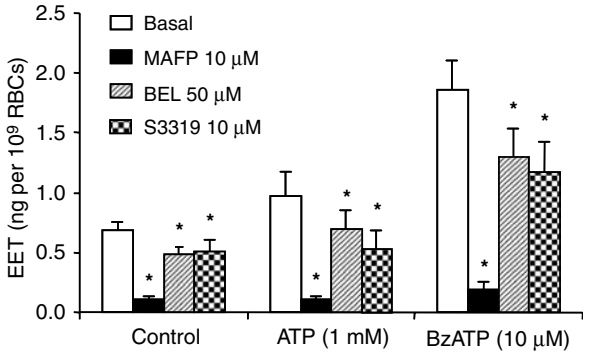

Figure 7 Effects of PLA_2 inhibitors, MAFP, BEL and S3319, on EET release from rat erythrocytes. MAFP, a Ca^{2+}-dependent cytosolic PLA_2 inhibitor, BEL, a Ca^{2+}-independent cytosolic PLA_2 inhibitor, and S3319, a secretory PLA_2 inhibitor were tested in the RBC incubations. $n=6$–8, $^*P<0.05$ vs basal in the same group. BEL, bromoenol lactone; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; EET, epoxyeicosatrienoic acid; MAFP, methyl arachidonyl flurophosphonate.

Discussion

There is compelling evidence that the erythrocyte acts as an oxygen sensor; namely, in response to physiological declines in oxygen tension, ATP released from erythrocytes acts on microvascular endothelial cells to produce vasodilatation (Dietrich *et al.*, 2000; Feigl, 2004). The present study expands the sphere of activity of ATP by showing that ATP, by stimulating erythrocyte $P2X_7$ receptors, releases EETs. As EETs exhibit a wide range of actions within the circulation, viz., vasodilation as well as antithrombotic and

Table 3 Comparison of the total EET release with AA release and haemolysis from rat erythrocytes (2×10^9 cells ml^{-1}, $n = 4$–10)

	Basal	*ATP (1 mM)*	*BzATP (10 μM)*	*Cu $(NO_3)_2$ (100 μM)*	*Freeze–thawing*
EET release	0.66±0.14	0.98±0.16*	1.87±0.20*	n.d.	0.35±0.12*
AA release	18.29±2.26	17.52±2.36	18.07±2.18	51.17±4.42*	55.32±6.15*
Haemolysis (mg l^{-1})	115±10	111±9	111±7	543±91*	13480±430*

Abbreviations: AA, arachidonic acid; BzATP, 2′(3′)-*O*-(4-benzoylbenzoyl)adenosine 5′-triphosphate; EET, epoxyeicosatrienoic acid; n.d., not detected.
Data are expressed as mean±s.e. EET and AA release are expressed as ng per10^9 RBCs.
*$P<0.05$ as compared with basal in the same category.

anti-inflammatory activities (Spector *et al*., 2004), they can account for many of the vascular responses to ATP.

The sequence of events in RBCs that follow upon ATP/BzATP stimulation of $P2X_7$ receptors is initiated by activation of PLA_2, which releases AA and EETs from storage in phospholipids (Salgo *et al*., 1993; Jiang *et al*., 2005). As deacylated AA can be converted to EETs by the epoxygenase-like activity of haemoglobin acting as a catalyst in RBCs (Table 1), the EETs secreted from RBCs in response to ATP/BzATP stimulation represent both release from storage in RBC phospholipids and *de novo* synthesis (Figure 1d). The secretion of EETs from RBCs involves both CFTR and pannexin-1 acting as conductive, that is, secretory pathways, for 'the selective delivery of nucleotides' and EETs 'to the extracellular domain' (Reisin *et al*., 1994). This secretory mechanism is based on the functional duality of the $P2X_7$ receptor that not only 'acts as an ATP-activated ion channel but also forms a pore, gating passage of molecules up to 1000 Da' such as EETs 'in response to ATP' (Lazarowski *et al*., 2003).

ATP at 5 μM and 1 mM increased EET release by *ca*. 20 and 40%, respectively (Figure 2), whereas at 10 μM, BzATP produced a threefold increase in EET release from RBCs (Figure 4). High concentrations of ATP (1 mM) were tested for EET release because of the relative insensitivity of RBC $P2X_7$ receptors to extracellular ATP (Sluyter *et al*., 2004; Young *et al*., 2007). Additionally, a P2Y receptor has been identified in RBCs that represents a negative feedback mechanism regulating erythrocyte ATP secretion (Wang *et al*., 2005). The role of P2Y receptors was uncovered by inhibition of EET release from the rat erythrocyte in response to UTP, the prototypical P2Y agonist (Kochukov and Ritchie, 2004; Figure 3). Additionally, increased EET release from RBCs produced by suramin at 100 μM (Table 2) can be explained by selective inhibition of P2Y receptors (King *et al*., 1996; Bourke *et al*., 1999) that exert a tonic inhibitory effect on EET release. In contrast, inhibition of EET release by RB-2 (Table 2), a nonselective ATP antagonist, is a function of its capacity to block all P2 receptors.

The EC_{50} value, 6.6 μM of BzATP for EET release (Figure 4) is in accordance with the reported EC_{50} for the rat $P2X_7$ receptor (Surprenant *et al*., 1996). The EC_{50} value for ATP on erythrocyte EET release could not be estimated because of the cited antagonism of P2Y receptors to $P2X_7$ receptor-mediated EET release. Spontaneous EET release from RBCs was inhibited by both $P2X_7$ antagonists, BBG and Zn^{2+} (Figure 5), suggesting a function for the RBC $P2X_7$ receptor related to basal formation and release of EETs.

Haemoglobin catalysed conversion of AA into EETs was inhibited by MSPPOH or ODYA (Table 1), suggesting an epoxygenase-like activity of haemoglobin. Thus far, an epoxygenase has not been identified in RBCs, but 'the mechanism and versatility of the monooxygenase activity of haemoglobin are qualitatively similar to those of the liver microsomal cytochrome *P*450' (Mieyal and Starke, 1994). In intact RBCs, the formation of EETs is regulated by $P2X_7$ receptor responses such as increases in cytoskeleton movement (Kim *et al*., 2001; Kochukov and Ritchie, 2004), phospholipid signalling (Garcia-Marcos *et al*., 2006) and oxidative burst (Suh *et al*., 2001; Parvathenani *et al*., 2003). Further, $P2X_7$ receptor stimulation by ATP and BzATP released EETs from RBCs without producing haemolysis, in contrast to the effects of A23187. $Cu(NO_3)_2$ and freeze–thawing produced haemolysis but had minimum or absent effect on EET release (Table 3).

CFTR activity in RBCs was linked to ATP release induced by deformation of erythrocytes (Sprague *et al*., 1998) as well as to ATP release produced by hypoxia (Bergfeld and Forrester, 1992). CFTR is a member of the ATP-binding cassette (ABC) family of proteins that 'drive the transport of various molecules across all cell membranes' (Dean *et al*., 2001). Glibenclamide, a sulphonylurea inhibiting potassium and chloride channels, blocks activation of the CFTR channel (Sheppard and Welsh, 1993; Schwiebert *et al*., 1995) and ATP release in response to shear stress in blood vessels (Hassessian *et al*., 1993). Niflumic acid, a cyclooxygenase and chloride channel inhibitor, also inhibits CFTR activity (Sheppard and Welsh, 1993; Cuthbert *et al*., 1994); both glibenclamide and niflumic acid reduced basal as well as ATP- and BzATP-stimulated release of EETs from erythrocytes (Figure 6).

The ATP secretory mechanism in erythrocytes has been described as enigmatic. It has taken on added complexity with the finding that the gap junction protein, pannexin-1, also functions as an ATP release channel in erythrocytes (Locovei *et al*., 2006). Pannexin-1, in addition to forming gap junctions, can form membrane channels that are mechanosensitive and exhibit high ATP permeability. Inhibition of EET release by the gap junction inhibitor, carbenoxolone (Figure 6), came as no surprise since pannexin-1 is a hemichannel associated with the $P2X_7$ receptor (Pelegrin and Surprenant, 2006). The common requirement of both CFTR and pannexin-1 activities for ATP and EET release from RBCs indicates an intrinsic association between the two transporters in erythrocytes that involves a regulatory ensemble including ABC transporters, a pannexin hemichannel and ATP-activated P2 receptors coupled to

G proteins (P2Y receptors) and ATP-gated cation channels (P2X receptors).

There are several aspects of the contribution of erythrocytes to regulation of vascular function that bear emphasizing. First, its magnitude, given the RBC as the predominant formed element of the blood with ATP concentrations several 1000-fold higher than those in plasma (Gonzalez-Alonso *et al*., 2002). Second, the abundance of EETs in the phospholipids of RBCs (Jiang *et al*., 2005) that are subject to rapid release in response to stimulation of RBC $P2X_7$ receptors with activation of PLA_2. Cytosolic Ca^{2+}-dependent PLA_2 has been identified in human (Macdonald *et al*., 2004) and bovine RBCs (Shin *et al*., 2002). Third, ATP exhibits a duality of effects on EET release, inhibiting release by stimulating P2Y receptors and activating release by stimulating $P2X_7$ receptors. The result is a multiphasic effect on EET release from RBCs in response to ascending concentration of ATP (Figure 2), whereas BzATP produced a curvilinear dose-dependent response on EET release (Figure 4).

In conclusion, the high levels of ATP in erythrocytes (Miseta *et al*., 1993) and the increasing recognition of the important role of ATP in regulating local blood flow (Dietrich *et al*., 2000) by stimulating endothelial purinoceptors (Stepp *et al*., 1996; Headrick *et al*., 2003), occasioned our studying the EET releasing mechanisms in RBCs. RBCs are reservoirs of EETs that are released by ATP activation of the $P2X_7$ receptor. EETs participate in regulating vasomotion in the coronary (Gauthier *et al*., 2005; Larsen *et al*., 2006), cerebral (Medhora *et al*., 2001) and renal (Imig *et al*., 2001; Pomposiello *et al*., 2003) vasculatures. As ATP release from RBCs is increased in response to deformation (Sprague *et al*., 1998), exercise (Farias III *et al*., 2005) and hypoxia (Dietrich *et al*., 2000), the association of EET release with that of ATP points to a role of erythrocyte-derived EETs in regulating local blood flow.

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Conflict of interest

The authors state no conflict of interest.

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