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Prostacyclin Analogues Stimulate Receptor-mediated cAMP Synthesis and ATP Release from Rabbit and Human Erythrocytes

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Abstract

Objectives—The purpose of this study was to establish that the prostacyclin (PGI_2) receptor (IP receptor) is present on rabbit and human erythrocytes and that its activation stimulates cAMP synthesis and ATP release.

Methods—The effect of incubation of erythrocytes with the active PGI_2 analogues, iloprost or UT-15C, on cAMP levels and ATP release was determined in the absence and presence of the IP receptor antagonist, CAY10441. Western analysis was used to determine the presence of the IP receptor on isolated membranes. To establish that effects of PGI_2 analogues were not due to prostaglandin E_2 (PGE_2) receptor activation, the effect of PGE_2 on cAMP levels and ATP release was determined.

Results—Rabbit and human erythrocytes possess IP receptors. Iloprost and UT-15C stimulated increases in cAMP and ATP release that were prevented by the IP receptor antagonist, CAY10441. PGE_2 did not stimulate cAMP accumulation or ATP release and did not inhibit iloprost-induced increases in cAMP.

Conclusions—This study establishes that the IP preceptor is present on rabbit and human erythrocytes and that its activation results in increases in cAMP and ATP release. These results suggest a novel mechanism by which PGI_2 and its active analogues, when administered pharmacologically, could produce vasodilation.

Keywords

Red blood cell; adenylyl cyclase; adenosine triphosphate; iloprost; UT-15C

INTRODUCTION

Prostacyclin (PGI_2), administered therapeutically, is a potent vasodilator in the pulmonary and systemic circulations. PGI_2 and its active analogues have been used successfully in the treatment of pulmonary hypertension in humans (21,28,30,40). Indeed, in idiopathic pulmonary arterial hypertension (IPAH), a human condition of unclear etiology and with high mortality, the administration of these agents is the most effective means of reducing pulmonary vascular resistance and prolonging survival (21,40). PGI_2 and its active analogues bind to the PGI_2 (IP) receptor which is coupled to Gs and adenylyl cyclase (7,14).

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The mechanism by which PGI_2 and its active analogues reduce pulmonary vascular resistance has been assumed to be via activation of the IP receptor on vascular smooth muscle cells resulting in increases in intracellular cAMP and, ultimately, vasodilation (14).

However, it has been shown that, in addition to the endothelial cell, another cell present in the circulation can contribute to the regulation of vascular caliber, namely, the erythrocyte. Erythrocytes, via their ability to release ATP, were shown to contribute to the maintenance of low vascular resistance in isolated perfused rabbit lungs (44,46) and to mediate vasodilation in response to exposure to reduced oxygen tension in isolated rat cerebral arterioles (9).

A signal transduction pathway that relates physiological and pharmacological stimuli to ATP release from rabbit and human erythrocytes has been described. Components of this pathway include the heterotrimeric G proteins Gs (38) and Gi (39) and adenylyl cyclase (42,44,46). Importantly, increases in cAMP are required for ATP release from these cells (45).

Human and rabbit erythrocytes have been shown to bind PGI_2 and PGE_1 in a highly selective, saturable and reversible fashion that is both concentration- and time- dependent (10,11,50). Although these findings suggest that PGI_2 binds to a receptor on the erythrocyte, the presence of the IP receptor on that cell has not been demonstrated. The only physiological consequence of the binding of PGI_2 to erythrocytes was reported to be an increase in erythrocyte deformability (23,24). In the absence of demonstrable PGI_2-associated activation of signaling pathways in the erythrocyte, it was suggested that the binding of PGI_2 by these cells could serve as a mechanism to decrease the bioavailability of PGI_2 permitting it to act locally while limiting systemic circulatory effects (50). In support of this alternative interpretation, when exposed to low nanogram concentrations of [^{3}H]-PGI_2 in plasma, erythrocytes rapidly bind nearly 80% of the available [^{3}H]-PGI_2 accelerating its conversion to the inactive degradation product, 6- keto-$PGF_{1\alpha}$ (50). Therefore, it is possible that through sequestration and degradation of pharmacologically administered PGI_2, the erythrocyte may prevent this PGI_2 from interacting with IP receptors on the vascular smooth muscle. However, if the binding of PGI_2 or its active analogues to erythrocytes results in receptor-mediated increases in cAMP leading to ATP release, this would provide a heretofore unrecognized mechanism by which these agents could participate in the regulation of vascular resistance when administered pharmacologically.

Here, we demonstrate, for the first time, that the IP receptor is a component of rabbit and human erythrocyte membranes. In addition, we show that the active PGI_2 analogues, iloprost (36) and UT-15C (treprostinil diethanolamine, United Therapeutics Corp.) (7), stimulate both cAMP accumulation and ATP release from these cells. Finally, we demonstrate that pretreatment of rabbit and human erythrocytes with the IP receptor antagonist, CAY10441 (8), prevents increases in cAMP and ATP release produced by iloprost and UT-15C.

MATERIALS AND METHODS

Isolation of rabbit and human erythrocytes

Rabbit blood was obtained from male New Zealand white rabbits (2–3 kg). Animals were anesthetized with ketamine (12.5 mg/kg) and xylazine (1.5 mg/kg) intramuscularly followed by pentobarbital sodium (10 mg/kg) intravenously. After tracheal intubation, animals were ventilated with room air (tidal volume; 10 ml/kg, rate; 25 breaths/min). A catheter was placed in a carotid artery for administration of heparin (500 units) and for blood removal. Ten min after heparin administration the animals were exsanguinated. Human blood was obtained from healthy volunteers by venipuncture (anticubital vein) and collected into a 35

ml syringe containing 50 units of heparin. The average age of the human subjects was 37±5 years (range 23 to 63). Eight male and 4 female subjects were studied. Protocols used to obtain blood from rabbits and humans were approved by the Institutional Animal Care and Use Committee and by the Institutional Review Board of Saint Louis University, respectively.

Blood was centrifuged at 500 × g at 4°C for 10 min and the plasma, buffy coat and upper layer of erythrocytes were removed by aspiration. The remaining erythrocytes were re-suspended and washed three times in wash buffer [in mM, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 1.2 $MgSO_4$, 21.0 tris(hydroxymethyl)aminomethane, 5.5 glucose with 0.5% bovine serum albumin (final pH 7.4)]. This procedure resulted in a concentrated preparation of erythrocytes with a hematocrit of 65 to 70%. Wright stains of erythrocytes prepared in this fashion reveal less than 1 leukocyte per 50 high power fields. Cells were prepared on the day of use and studied within 3 hours of collection and washing.

Preparation of rabbit and human erythrocyte membranes

All procedures were performed at 4°C. Washed erythrocytes (2 ml) were added to 200 ml of hypotonic buffer (in mM; 5 Tris-HCl, 2 EDTA with pH adjusted to 7.4) and stirred vigorously for 20 min. The mixture was centrifuged at 23,300 × g for 15 min. The supernatant was discarded and the membranes were re-suspended in hypotonic buffer, centrifuged at 23,300 × g for 15 min and the supernatant again discarded. This procedure was repeated a third time and the washed membranes were pooled. The protein concentration of membrane preparations was determined with the BCA Protein Assay (Pierce). Aliquots were stored at −80°C.

Preparation of rabbit and human platelet membranes

All procedures were performed at 4°C. Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 400 × g for 10 min. The PRP was collected and, after addition of 500 units of heparin and EDTA (1 mg/ml), was centrifuged again (200 × g for 40 min). The supernatant was collected and centrifuged at 1400 × g for 20 min. The resulting platelet pellet was lysed in 200 μl of buffer [in mM, 300.0 NaCl, 10.0 EDTA, 1.5 $MgCl_2$, 20 β-glycerophosphate, 0.1 sodium vanadate, 25.0 hepes, 1 ml Triton X-100 (final pH 7.4)] and centrifuged at 14,000 × g for 20 min. The protein concentration of the supernatant was determined with the BCA Protein Assay. Aliquots were stored at −80°C.

Measurement of cAMP

For determination of cAMP, erythrocytes were diluted with wash buffer (described above) to achieve a hematocrit of 50%. After incubation with pharmacological agents, 1 ml of the erythrocyte suspension was added to 4 ml of ice cold absolute ethanol containing HCl (1 mM) and the mixture was centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was removed and stored overnight at −20°C to precipitate remaining proteins. Samples were centrifuged a second time at 3,700 × g for 10 min at 4°C. The supernatant was retained and dried under vacuum centrifugation. Concentrations of cAMP were determined using enzyme immunoassay (GE Healthcare). Cell counts were obtained from the suspension of washed erythrocytes and amounts of cAMP measured were normalized to 10^{10} cells/ml.

Measurement of ATP and hemoglobin

ATP was measured by the luciferin-luciferase technique (11,12,14–19) which utilizes the ATP concentration-dependence of light generated by the reaction of ATP with firefly lantern extract. Sensitivity was augmented by addition of synthetic D-luciferin to the crude firefly lantern extract. A 200 μl sample of erythrocyte suspension (0.04 % hematocrit) was injected

into a cuvette containing 100 μl crude firefly lantern extract (10 mg/ml distilled water, FLE 250) (Sigma) and 100 μl of a solution of synthetic D-luciferin (50 mg/100 ml distilled water) (Sigma). The light emitted was detected using a luminometer (Turner Designs). A standard curve was obtained on the day of each experiment. To exclude the presence of significant hemolysis, samples were centrifuged at 500 × g at 4°C for 10 min and the presence of free hemoglobin in the supernatant was determined by light absorption at a wavelength of 405 nm. All data were excluded from experiments in which free hemoglobin was detected. Cell counts were obtained from the suspension of washed erythrocytes and amounts of ATP measured were normalized to 4×10^8 cells/ml.

Concentration-response relationship for PGI_2 analogue-induced increases in cAMP in rabbit and human erythrocytes

Washed erythrocytes of rabbits or humans (50% hematocrit) were incubated with either iloprost (rabbit and human erythrocytes) (Cayman Chemical Company) or UT-15C (rabbit erythrocytes) (United Therapeutics Corp.) at concentrations of 10 nM to 1 μM. Amounts of cAMP were determined at baseline and 15 min after addition of the prostacyclin analogues or their vehicle (saline).

Western analysis of erythrocyte and platelet membranes

Erythrocyte membranes (50 or 100 μg of protein for rabbits and humans, respectively) or platelet membranes (0.1 or 0.03 μg of protein for rabbits and humans, respectively) were solubilized in sample buffer (0.277 M sodium dodecyl sulfate (SDS), 60% glycerol, 0.4 M dithiothreitol, 0.25 M Tris HCl and 0.004% bromophenol blue), heated (5 min, 100°C) and loaded onto a precast 4–12% gradient or 7.5% SDS-PAGE gel (Cambrex) and subjected to electrophoresis in buffer containing 25 mM Tris, 192 mM glycine and 0.1% w/v SDS, pH 8.3 at 4°C. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane in transfer buffer (25 mM Tris and 192 mM glycine with 20% v/v methanol at pH 8.3) at 4°C. PVDF membranes were then incubated with an antibody directed against the human IP receptor (rabbit polyclonal, Cayman Chemical Company, 1:800 dilution). On separate gels, the same membrane preparations were incubated with antibody directed against human CD41 (mouse monoclonal, Chemicon, 1:1000 dilution). Platelet membranes were included on the latter gels as a positive control. After incubation with the primary antibodies, membranes were incubated with appropriate secondary antibodies and exposed to enhanced chemiluminescence using ECL (Pierce).

Determination of cAMP levels of erythrocytes in response to incubation with IP receptor agonists, isoproterenol or PGE_2 in the absence and presence of receptor antagonists

Washed rabbit erythrocytes (50% hematocrit) were incubated with eitheriloprost (1 μM), UT-15C (1 μM), isoproterenol (ISO, 1 μM) (Sigma) or PGE_2 (1 μM) (Sigma). The agonists were administered in the presence and absence of a 30 min pretreatment with either an IP receptor antagonist (CAY10441, 10 μM) (Cayman Chemical Company) or a β-adrenergic receptor antagonist, propranolol (10 μM) (Sigma). In separate studies, washed human erythrocytes, were incubated with iloprost in the absence and presence of CAY10441. In all studies, cAMP concentration was determined 15 min after addition of agonist.

Determination of ATP release from erythrocytes in response to incubation with IP receptor agonists in the absence and presence of CAY10441

Washed rabbit erythrocytes (20% hematocrit) were incubated with either UT-15C (1 μM) in the absence or presence of the IP receptor antagonist (CAY10441, 10 μM). In separate studies human erythrocytes were incubated with iloprost (1 μM) in the absence or presence of CAY10441 (10 μM). The ATP concentration in the cell suspension was determined 30

min after addition of CAY10441 and at 5, 10 and 15 min after addition of iloprost and at 1, 5 and 10 min after addition of UT-15C. These time periods were chosen based on preliminary studies. The response to each agonist is reported as the maximal response observed. The maximum increase in ATP release in response to iloprost occurred at 11±2 and 10±1 min in rabbit and human erythrocyte preparations, respectively. The maximum response to UT-15C occurred at 5±1 min in rabbit erythrocyte preparations. In addition to stimulated ATP release, total ATP content of erythrocytes in all groups was determined by lysis of a known number of erythrocytes.

Statistical methods

Statistical significance was determined with an analysis of variance (ANOVA). If the *F* ratio indicated that changes occurred, a Fischer's LSD protected *T*-test was used to identify individual differences. A *p* value of 0.05 or less was considered to be statistically significant. Results are reported as means ± SE.

RESULTS

Effect of prostacyclin analogs on cAMP levels in rabbit and human erythrocytes

Incubation of rabbit erythrocytes with either iloprost (n=4) or UT-15C (n=4) resulted in concentration-dependent increases in cAMP (fig 1, A). Human erythrocytes also displayed concentration-dependent increases in cAMP when incubated with iloprost (fig 1, B), although the maximum response was greater.

Identification of the IP receptor as a component of rabbit and human erythrocyte membranes

To establish that IP receptors are present on rabbit and human erythrocytes, isolated membranes were resolved by Western analysis and probed with an antibody generated against amino acids 1–16 of the N-terminal portion of the human IP receptor. This antibody identified a band with the predicted molecular mass of the IP receptor in membranes of rabbits and humans (fig 2, A&B). The gels are representative of studies with seven rabbit and fourteen human erythrocyte membrane preparations. In order to ensure that the erythrocyte membrane preparations were not contaminated with platelets, formed elements of the blood that also express the IP receptor (27), separate studies were performed in which the same membrane preparations were probed with an antibody directed against CD41, a protein expressed in platelets (27). As shown in figure 3, there was no significant platelet contamination of human erythrocyte membrane preparations as indicated by the inability to detect CD41. Two commercially available antibodies to CD41 did not bind to the rabbit CD41 antigen. However, manual platelet counts performed on whole rabbit blood revealed platelet counts in excess of 300,000/mm^3 while no platelets were detected in the washed erythrocyte preparations. These findings indicate that platelet contamination does not account for the detection of the IP receptor in erythrocyte membrane preparations from either species.

Effect of an IP receptor antagonist on PGI_2 analogue-induced increases in cAMP in rabbit and human erythrocytes

Incubation of washed rabbit erythrocytes with iloprost (1 μM, n=9) resulted in a 171±32% increase in cAMP content (fig 4, A). Pretreatment of rabbit erythrocytes with the IP receptor antagonist, CAY10441 (10 μM, n=5), had no effect on basal cAMP levels but prevented the iloprost-induced increase in cAMP (fig 4, A). The vehicle for CAY10441 (N′,N-dimethylformamide, DMF) had no effect on cAMP levels in erythrocytes (n=6). To demonstrate that CAY10441 did not inhibit the ability of erythrocytes to synthesize cAMP,

rabbit erythrocytes were incubated with the β-adrenergic receptor agonist, isoproterenol (1 μM), in the absence and presence of CAY10441 and cAMP levels were determined. Isoproterenol (n=9) stimulated a 93±29% increase in cAMP that was not inhibited by pretreatment with CAY10441 (n=4) (fig 4, B). Importantly, pretreatment of rabbit erythrocytes with the β-receptor antagonist, propranolol (10 μM, n=5) inhibited isoproterenol-induced increases in cAMP but did not alter the response to iloprost (n=4) (fig 4, A and B). In separate studies, incubation of rabbit erythrocytes with a second IP receptor agonist, UT-15C (1 μM, n=9) resulted in an increase in cAMP of 189±38% that was prevented by pretreatment with CAY10441 (fig 5, B). To establish that the ability of an IP agonist to increase cAMP levels was not limited to erythrocytes of rabbits, human erythrocytes were incubated with iloprost (1 μM, n=5) in the absence and presence of CAY10441. As shown in figure 5B, iloprost produced a 168 ±72% increase in cAMP that was prevented by pretreatment with CAY10441.

It has been reported that iloprost (25) and UT-15C (1), in addition to activation of IP receptors, can interact with members of another group of eicosanoid receptors, members of the EP receptor family. To establish that the increases in cAMP associated with incubation of erythrocytes with these PGI_2 analogues were not related to EP receptor activation, in separate studies, rabbit erythrocytes were incubated with the preferred agonist for EP receptors, PGE_2 (1 μM, n=4) or its vehicle, DMF. PGE_2 did not produce increases in cAMP nor did pretreatment with PGE_2 alter the response to iloprost (fig 6).

Effect of iloprost and UT-15C on ATP release from erythrocytes in the absence and presence of an IP receptor antagonist

Incubation of rabbit erythrocytes with UT-15C (1 μM, n=9) resulted in a 334±137% increase in ATP release (fig 7,A). This release of ATP was prevented by pretreatment of rabbit erythrocytes with CAY10441 (10 μM, n=7, fig 7,A). In separate studies, human erythrocytes were incubated with iloprost (1 μM) in the absence (n=7) and presence (n=5) of CAY10441. Iloprost produced a 332±72% increase in ATP release that was prevented in the presence of the IP receptor antagonist (fig 7,B). To ensure that decreased ATP release did not reflect depletion of intracellular ATP by CAY10441, levels of total intracellular ATP in the absence and presence of the inhibitor were determined. As shown in table 1, total intracellular ATP levels in rabbit and human erythrocytes were not affected by incubation with CAY10441. Finally, incubation of rabbit (n=5) and human (n=5) erythrocytes with PGE_2 did not result in ATP release. Thus, the ATP release produced by PGI_2 analogues was not the result of EP receptor activation.

DISCUSSION

The erythrocyte, by virtue of the hemoglobin that it contains, has long been recognized as a vehicle for oxygen transport. In addition to this well established function, it has been shown that this cell can participate in the local regulation of vascular resistance via its ability to release ATP (3,5,9,12,13,44). ATP released from erythrocytes can interact with purinergic receptors on the vascular endothelium (4,15,18) resulting in the synthesis and release of vasodilators including nitric oxide (NO) and metabolites of arachidonic acid (5,15,44,46). A signal transduction pathway that relates physiological and pharmacological stimuli to ATP release from erythrocytes has been defined. This pathway includes the heterotrimeric G proteins Gs (38) and Gi (39), adenylyl cyclase (AC) (40,43,45), protein kinase A (PKA) (45), as well as the cystic fibrosis transmembrane conductance regulator (43). Importantly, increases in cAMP are required for ATP release from human and rabbit erythrocytes (45).

Although older reports had suggested that human erythrocytes were nearly devoid of AC activity, the techniques used to isolate erythrocyte membranes in those studies likely

damaged the enzyme dramatically decreasing its ability to synthesize cAMP (35). More recently it was shown that rabbit and human erythrocytes possess AC activity (35,41,45) and, using Western analysis, the presence of type II AC in rabbit and human erythrocytes was established (42,47). Support for a functional role for cAMP in erythrocyte physiology was demonstrated in studies in which forskolin, a non-selective activator of AC as well as the active cAMP analogue, Sp-cAMP, stimulated ATP release from rabbit and human erythrocytes (45). In addition, it was shown that human erythrocytes possess β-adrenergic receptors and that incubation of these cells with the β-agonist isoproterenol resulted in increases in intracellular cAMP (35,41,42)

In addition to β agonists, PGI_2 and PGE_1 also bind to human erythrocytes in a highly selective, saturable and reversible fashion (10,11,50). However, in spite of the fact that this binding was shown to be concentration- and time-dependent, the only reported physiological consequence of PGI_2 binding was an increases in erythrocyte deformability (23,24). Thus, although previous studies demonstrated that PGI_2 and PGE_1 interact with erythrocytes in a manner consistent with receptor binding, in the absence of evidence demonstrating activation of intracellular signaling pathways, it was suggested that this binding represented a mechanism for the *in vivo* inactivation and metabolism of PGI_2 (50).

Here, we report that incubation of erythrocytes of rabbits and humans with active PGI_2 analogues, iloprost or UT-15C, results in concentration-dependent increases in cAMP (fig 1) and ATP release (fig 7), results consistent with activation of a PGI_2 receptor. We next determined if these effects of iloprost and UT-15C were, in fact, mediated via activation of IP receptors on rabbit and human erythrocytes. Using Western analysis, we determined that the IP receptor is present in membranes of erythrocytes of both species (fig 2). Using an antibody to human CD41, a protein found on platelets but not on erythrocytes (27), we were unable to detect any platelet contamination of human erythrocyte membrane preparations (fig 3). In addition, no platelets were detected by manual counting of washed rabbit erythrocyte preparations from which membranes were isolated. These results support the hypothesis that concentration-dependent increases in cAMP as well as ATP release produced by iloprost and UT-15C are the consequence of activation of IP receptors on rabbit and human erythrocytes.

The mature erythrocyte lacks a nucleus and protein synthetic machinery preventing the use of techniques to alter IP receptor expression in these cells. However, a selective antagonist of the IP receptor, CAY10441, has been developed (8). Pretreatment of erythrocytes of rabbits (fig 4,A) and humans (fig 5,A) with CAY10441 prevented iloprost-induced increases in cAMP. In addition, in rabbit erythrocytes, CAY10441 prevented increases in cAMP produced by a second chemically dissimilar IP receptor agonist, UT-15C (fig 5,B). To ensure that CAY10441 did not interfere directly with the ability of erythrocytes to synthesize cAMP, rabbit erythrocytes were treated with isoproterenol in the absence and presence of the IP receptor antagonist. CAY10441 had no effect on either basal cAMP levels (fig 4A) or isoproterenol-induced increases in cAMP (fig 4,B). The effect of isoproterenol, but not that of iloprost, was prevented by pretreatment with the β-adrenergic receptor antagonist, propranolol (fig 4, A&B). Thus, both CAY10441 and propranolol demonstrate selectivity and efficacy for their individual receptors. Finally, incubation of human erythrocytes with iloprost (fig 5,A) or rabbit erythrocytes with UT-15C (fig 5,B), produced increases in cAMP that were prevented also by CAY10441 pretreatment. Importantly, CAY10441 pretreatment also prevented increases in ATP released produced by incubation of rabbit and human erythrocytes with the active PGI_2 analogues (fig 7). Taken together, these results provide strong support for the hypothesis that iloprost and UT-15C produce increases in cAMP and ATP release from rabbit and human erythrocytes via activation of the IP receptor.

It was reported that both iloprost and UT-15C, in addition to binding to the IP receptor, can interact with other eicosanoid receptors that are members of the EP family (36). Four subtypes of the EP receptor have been identified. The preferred agonist for all members of this receptor family is PGE_2, an eicosanoid that does not activate the IP receptor (36). It has been suggested that erythrocytes possess EP receptors based on reports that PGE_2 stimulates activity of the calcium-dependent potassium channel (Gardos channel) in these cells (20,29). To determine if the effects of iloprost or UT-15C were a consequence of interaction with EP receptors, we examined the ability of PGE_2 to stimulate cAMP accumulation and ATP release from erythrocytes. Incubation with PGE_2 did not produce increases in cAMP in rabbit erythrocytes nor did pretreatment with PGE_2 diminish iloprost-induced increases in cAMP (fig 6). In addition, PGE_2 did not stimulate ATP release from rabbit or human erythrocytes. Thus, it is highly unlikely that iloprost- and UT-15C-induced increases in cAMP levels or ATP release from erythrocytes are mediated by activation of EP receptors.

The intravenous administration of either PGI_2 orits active analogues, iloprost and treprostinil, are effective treatments of pulmonary hypertension in humans (21,28,30,40). Long term continuous infusion of PGI_2 (epoprostenol) is associated with decreased pulmonary vascular resistance and increased survival in humans with idiopathic pulmonary arterial hypertension (IPAH) (21,40). It is well documented that PGI_2 and its active analogues produce vasodilation of isolated blood vessels via binding to IP receptors on the vascular smooth muscle resulting in increases in cAMP (14). However, when infused into humans or added to washed erythrocytes, the vast majority of PGI_2 is bound by erythrocytes (10,11,50). The finding that iloprost and UT-15C stimulate cAMP accumulation and ATP release from rabbit and human erythrocytes suggests a heretofore unrecognized mechanism by which the intravenous administration of PGI_2 could reduce pulmonary vascular resistance. ATP has been shown to produce vasodilation in isolated lungs via stimulation of NO synthesis (44). When PGI_2 or its active analogues are infused into the pulmonary circulation, their binding to IP receptors on erythrocytes would result in increases in cAMP and the release of ATP from that cell. ATP released from erythrocytes can then interact with endothelial purinergic receptors resulting in the release of NO. In support of this hypothesis, it was reported that humans with IPAH demonstrate decreased levels of NO in exhaled air when compared to healthy human control subjects (21,40). However, in the IPAH patients receiving intravenous PGI_2, exhaled NO levels were greater than in IPAH patients not receiving PGI_2 or healthy control subjects (40). Although other interpretations are possible, these findings are consistent with the hypothesis described here, i.e., that intravenous infusion of PGI_2 in IPAH patients results in stimulation of ATP release from erythrocytes leading to increases in pulmonary endothelial NO synthesis. In light of the evidence supporting extensive binding and rapid degradation of PGI_2 in plasma containing washed human erythrocytes, it could be argued that PGI_2-induced release of ATP from erythrocytes, with subsequent ATP-induced activation of endothelial NO synthesis, may actually represent a more important mechanism by which exogenously administered PGI_2 opposes pulmonary hypertension than via a direct effect on vascular smooth muscle.

In summary, we have shown that the IP receptor is a component of the membranes of erythrocytes of humans and rabbits and that incubation of these cells with iloprost or UT-15C, two active PGI_2 analogues, stimulates receptor-mediated cAMP accumulation and ATP release. These results suggest a novel mechanism by which PGI_2 and its active analogues, when administered pharmacologically to intact animals, can produce vasodilation. Moreover, these findings suggest that the erythrocyte is a potential target for the design of new therapies for the treatment of pulmonary hypertension.

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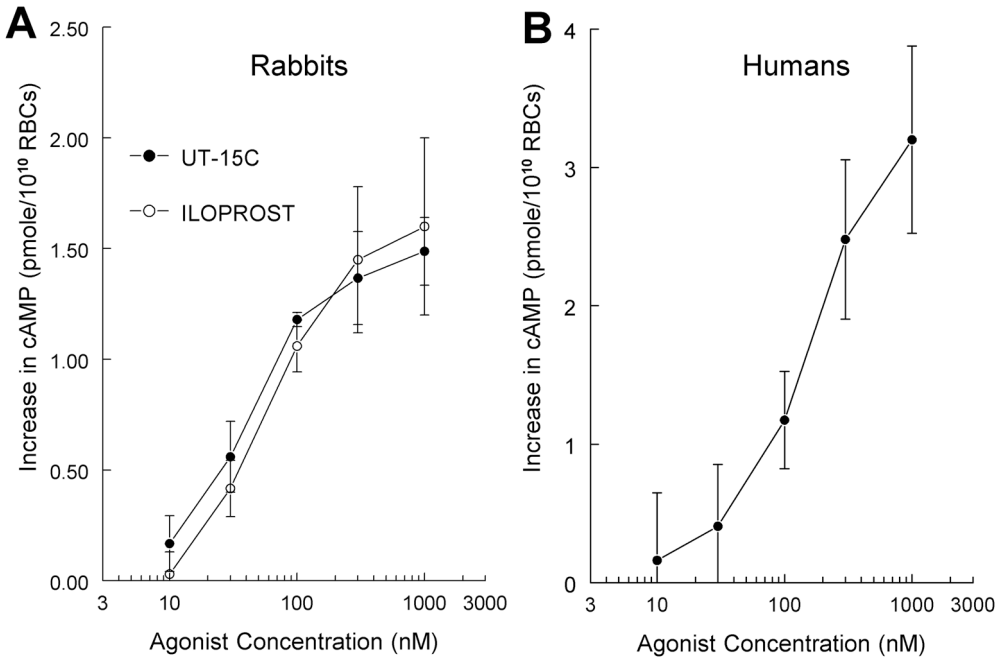

Figure 1.

A: Effect of iloprost (*n* = 4, open circles) and UT-15C (*n* = 4, closed circles) on increases in cAMP in rabbit erythrocytes (RBCs). Values are not different between agonists at any concentration. *B*: Effect of iloprost (*n* = 4) on increases in cAMP in human RBCs. Values are means ± SE.

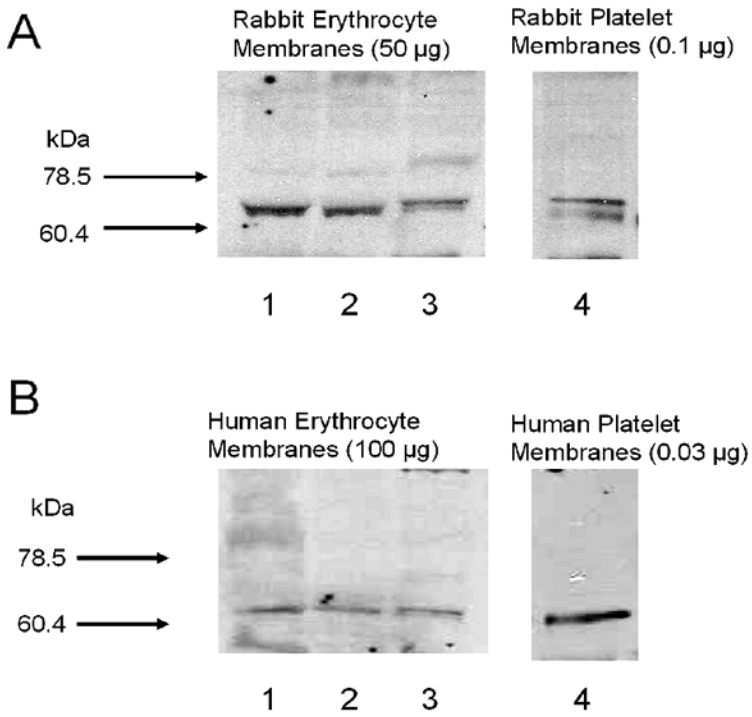

Figure 2.

Western immunoblots of rabbit (A) and human (B) erythrocyte membranes and platelet membranes (positive controls) probed with an antibody directed against the IP receptor. *A*: Lanes 1–3 = individual rabbit erythrocyte membrane preparations (representative of 7 studies) and lane 4 = rabbit platelet membranes. *B*: Lanes 1–3 = individual human erythrocyte membranes (representative of 14 studies) and lane 4 = human platelet membranes.

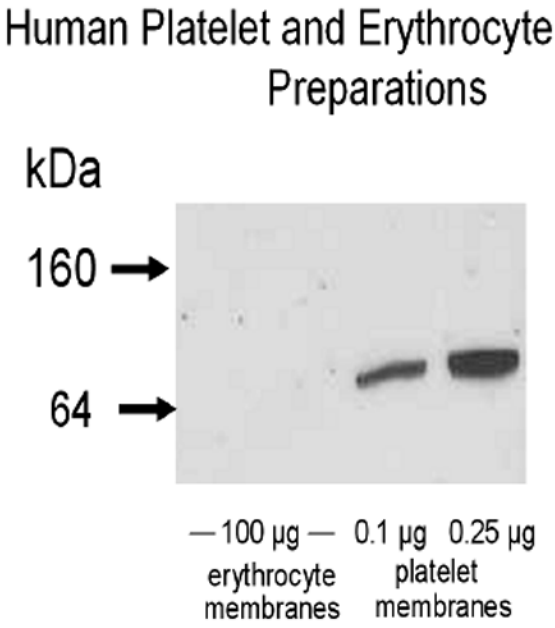

Figure 3.
Western immunoblot of human erythrocyte and platelet membranes probed with an antibody directed against CD41. Representative of studies with 7 human erythrocyte membrane preparations.

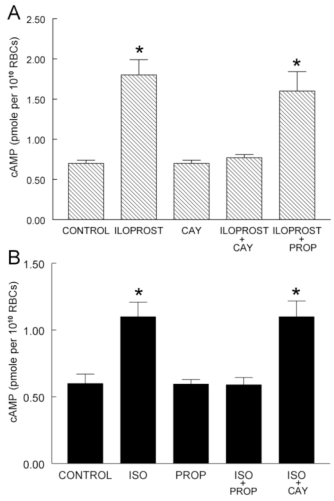

Figure 4.

A: Effect of iloprost (1 μM, *n* = 9) or its vehicle (CONTROL, saline, *n* = 9) on cAMP levels in rabbit erythrocytes. In some studies erythrocytes were incubated with the IP receptor antagonist CAY10441 alone (CAY, 10 μM, *n* = 5) or were preincubated with CAY10441 (*n* = 5) or the β-receptor antagonist, propranolol (PROP, 10 μM, *n* = 4) before the addition of iloprost. *B*: Effect of isoproterenol (ISO, 1 μM, *n* = 9) or its vehicle (CONTROL, saline, *n* = 9) on cAMP levels in rabbit erythrocytes. In some studies erythrocytes were incubated with the β-receptor antagonist, propranolol alone (PROP, 10 μM, *n* = 5) or were preincubated with PROP (*n* = 5) or the IP receptor antagonist CAY10441 (CAY, 10 μM, *n* = 4) before the addition of ISO. Values are means ± SE. **p* < 0.01 compared to all other groups.

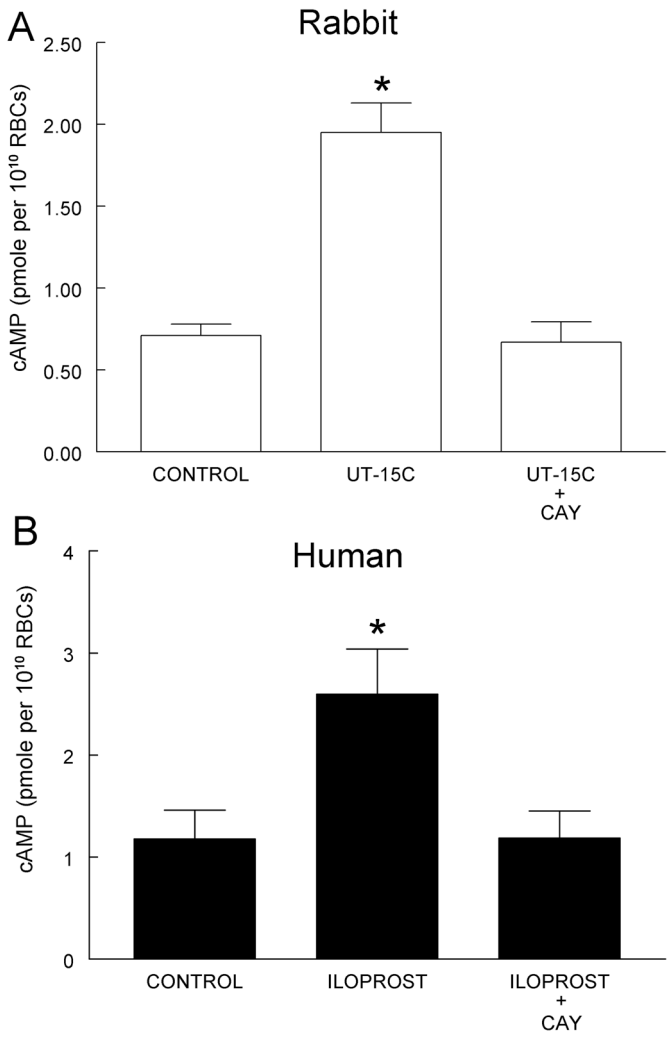

Figure 5.

A: Effect of UT-15C (1 μM, $n = 9$) or its vehicle (CONTROL, saline) on cAMP levels in rabbit erythrocytes in the absence and presence of the IP receptor antagonist CAY10441 (CAY, 10 μM). *B*: Effect of iloprost (1 μM, $n = 5$) or its vehicle (CONTROL, saline) on cAMP levels in human erythrocytes in the absence and presence of the IP receptor antagonist CAY10441 (CAY, 10 μM). Values are means ± SE. *$p < 0.01$ compared to all other groups.

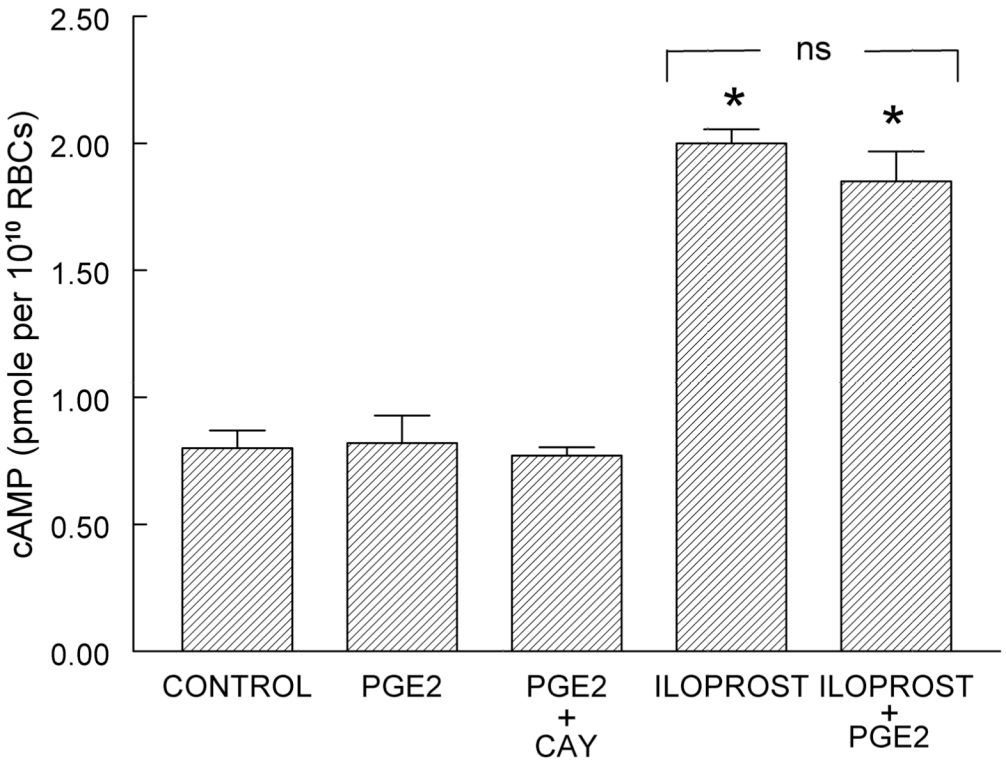

Figure 6.
Effect of prostaglandin E_2 (PGE_2, 1 μM, $n = 4$) on cAMP levels in rabbit erythrocytes in the absence and presence of the IP receptor antagonist CAY10441 (CAY, 10 μM) or iloprost (1 μM). Values are means ± SE. ns = not significantly different. *$p < 0.01$ compared to all other groups.

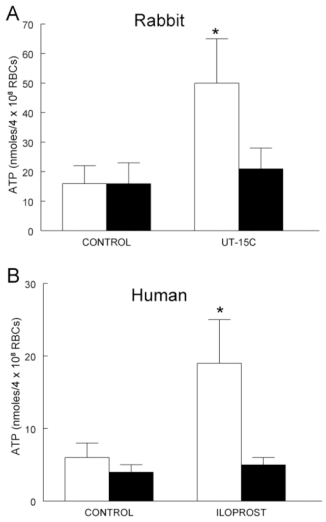

Figure 7.

A: Effect of UT-15C (1 μM) or its vehicle (CONTROL, saline) on ATP release from rabbit erythrocytes in the absence (open bars, $n = 9$) and presence (closed bars, $n = 7$) of the IP receptor antagonist CAY10441 (CAY, 10 μM). *B*: Effect of iloprost (1 μM) or its vehicle (CONTROL, saline) on ATP release from human erythrocytes in the absence (open bars, $n = 7$) and presence (closed bars, $n = 5$) of the IP receptor antagonist CAY10441 (CAY, 10 μM). Values are means ± SE. *$p < 0.05$ compared to CONTROL.

Table 1

Concentration of adenosine triphosphate (ATP) in erythrocytes of rabbits and humans in the absence (BASELINE) and presence of the IP receptor antagonist, CAY10441.

	TOTAL ATP (mM per erythrocyte)	
GROUP	**BASELINE**	**CAY10441**
RABBITS	2.56±0.13 (n=11)	3.03±0.29 (n=5)
HUMANS	2.76±0.47 (n=4)	2.77±0.16 (n=4)

Values are mean ± SEM.