Synergistic effects of prostacyclin analogs and phosphodiesterase inhibitors on cyclic adenosine 3′,5′ monophosphate accumulation and adenosine 3′5′ triphosphate release from human erythrocytes

Stephanie M Knebel, Mollisa M Elrick, Elizabeth A Bowles, Amber K Zdanovec, Alan H Stephenson, Mary L Ellsworth and Randy S Sprague

Department of Pharmacological and Physiological Science, Saint Louis University, St. Louis, MO 63104, USA
Corresponding author: Stephanie M Knebel. Email: knebelsm@slu.edu

Abstract

Prostacyclin (PGI_2) and phosphodiesterase 5 (PDE5) inhibitors are potent vasodilators that are used alone and in combination for the treatment of pulmonary arterial hypertension (PAH). Although these vasodilators are known to stimulate relaxation of vascular smooth muscle directly, other cells in circulation, including erythrocytes, express prostacyclin receptor (IPR) and contain PDE5. The binding of PGI_2 analogs to the erythrocyte IPR results in activation of a signaling pathway that increases cyclic adenosine 3′,5′ monophosphate (cAMP), a requirement for adenosine 3′5′ triphosphate (ATP) release. Within this pathway, cAMP levels are regulated by phosphodiesterase 3 (PDE3), a PDE that is inhibited by cGMP, a cyclic nucleotide regulated by the activity of PDE5. Since inhibition of PDE3 enhances ATP release in response to PGI_2 analogs, we investigated if the selective PDE5 inhibitors, zaprinast (ZAP) and tadalafil (TAD), would similarly increase cAMP and ATP release from human erythrocytes in response to the same stimulus. We determined that pretreatment of erythrocytes with one of two chemically dissimilar PDE5 inhibitors (ZAP or TAD, 10 μM) potentiated increases in cAMP and ATP release in response to incubation of human erythrocytes with the PGI_2 analog, UT-15C (100 nM). These results suggest that a heretofore unrecognized synergism exists between IPR agonists and PDE5 inhibitors that could provide a new rationale for the co-administration of these agents as vasodilators in humans with PAH.

Keywords: Phosphodiesterase 5, iloprost, UT-15C, tadalafil, zaprinast

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Introduction

Pulmonary arterial hypertension (PAH) is a chronic disorder characterized by sustained increases in pulmonary vascular resistance leading to pulmonary hypertension and right ventricular heart failure.[1,2] Although the pathophysiology of PAH is not fully understood, it is known that some forms of PAH have a hereditary component, while the majority occur spontaneously or are associated with other disease processes such as connective tissue disease or chronic liver disease.[3,4] Etiology notwithstanding PAH has a poor prognosis in the absence of pharmacological intervention. The major classes of drugs used to treat severe PAH include endothelin receptor antagonists, prostacyclin (PGI_2) analogs and inhibitors of phosphodiesterase 5 (PDE5).[1,2]

It is widely accepted that PGI_2, as well as its analogs, dilates blood vessels by stimulating prostacyclin receptor (IPR)-mediated increases in cyclic adenosine 3′,5′ monophosphate (cAMP) in vascular smooth muscle cells.[5–7] However, human erythrocytes also express functional IPRs, and incubation of these cells with PGI_2 analogs activates a well-defined signalling pathway that culminates in the release of the vasoactive molecule, adenosine 3′5′ triphosphate (ATP).[8,9] When released from circulating erythrocytes in the vascular lumen, ATP binds to purinergic receptors on the endothelium of both the pulmonary and systemic circulations resulting in the synthesis of vasodilators, including nitric oxide.[10–14] Thus, PGI_2 or its analogs, when administered intravenously, can stimulate vasodilation via direct effects on vascular smooth muscle, as well as indirectly by promoting the release of ATP from circulating erythrocytes.[6,15,16]

In erythrocytes, IPR-mediated ATP release requires increases in intracellular cAMP.[9] Since cAMP is a component of numerous and diverse signalling pathways in cells, its concentration must be localized and tightly regulated to

allow for discrete responses to different stimuli.[17] In all signalling pathways, local levels of cAMP are the product of its synthesis by adenylyl cyclases and its hydrolysis by phosphodiesterases (PDEs).[17,18] A critical component responsible for the localization of cAMP signalling to a given pathway is the activity of pathway-specific PDEs.[19,20]

The cAMP hydrolyzing PDEs 2, 3 and 4 have been identified in human erythrocytes and are associated with distinct receptor-mediated signalling pathways for ATP release.[8,21,22] In these cells, PDEs 2 and 4 are involved in the hydrolysis of cAMP in the ATP release pathway initiated by the activation of β-adrenergic receptors[21] while PDE3 is responsible for cAMP hydrolysis in the pathway initiated by the activation of IPR.[22] In addition, PDE5, a cGMP-specific PDE, has been identified in cytosolic fractions of human erythrocytes, and its inhibition increases cGMP levels in these cells.[23] Importantly, since PDE3 is inhibited by cGMP,[24,25] increases in cGMP in erythrocytes could inhibit PDE3 activity and augment IPR-mediated increases in cAMP leading to increases in ATP release.

The overall aim of this study was to investigate if increases in cAMP and ATP release from human erythrocytes in response to the PGI_2 analogs, iloprost and UT-15C (a treprostinil formulation that is water soluble) are potentiated by PDE5 inhibitors. The results provide the first evidence of a synergism between PGI_2 analogs and PDE5 inhibitors that result in enhanced IPR-mediated ATP release from erythrocytes. Such a relationship would suggest a new mechanism by which these drugs are effective in the treatment of PAH.

Materials and methods

Isolation of human erythrocytes

Blood was obtained from 32 healthy volunteers (13 males; average age 43 ± 4; range, 26–62; 19 females; average age 36 ± 3; range, 19–62) by venipuncture using a syringe-containing heparin (500 U). Informed consent was obtained from all subjects, and the protocol for blood removal was approved by the Institutional Review Board of Saint Louis University.

After collection, whole blood was centrifuged at $500 \times g$ at 4°C for 10 min, and the plasma, buffy coat and uppermost layer of erythrocytes were removed by aspiration and discarded. The remaining erythrocytes were washed three times in buffer containing 21.0 mM tris(hydroxymethyl) aminomethane, 4.7 mM KCl, 2.0 mM $CaCl_2$, 140.5 mM NaCl, 1.2 mM $MgSO_4$, 5.5 mM glucose and 0.5% bovine albumin fraction V (BSA), final pH 7.4. Erythrocytes prepared as described are devoid of leukocyte and platelet contamination.[22] Erythrocytes were isolated on the day of use.

Measurement of ATP release from human erythrocytes in response to PGI_2 analogs in the absence and presence of PDE inhibitors

Washed erythrocytes were diluted to a 20% hematocrit with the buffer described above. Erythrocytes were incubated with one of two chemically dissimilar PGI_2 analogs, UT-15C (United Therapeutics, Research Triangle Park, NJ, USA) or iloprost (Cayman Chemical, Ann Arbor, MI, USA). ATP release in response to administration of a PGI_2 analog was measured in the absence and presence of selective PDE inhibitors or their vehicle, dimethylformamide (DMF, Sigma, St. Louis, MO, USA). UT-15C-induced ATP release was determined in the absence and presence of one of two chemically dissimilar PDE5 inhibitors, zaprinast (ZAP, Sigma) or tadalafil (TAD, Eli Lilly, Indianapolis, IN, USA) while iloprost-induced ATP release was determined in the absence and presence of the PDE3 inhibitor, cilostazol, (CILO, 100 μm, Sigma). ATP was measured by the luciferin–luciferase technique[26] at 5, 10 and 15 min after the addition of a PGI_2 analog. The peak response is reported. A 200-μL sample of the erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 μL of 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; Research Products International, Mt. Prospect, IL, USA). The light emitted was detected using a luminometer (Turner Designs, Sunnyvale, CA, USA). A standard curve was obtained for each experiment. Cell counts were obtained from the suspension of erythrocytes. Amounts of ATP are reported as nanomoles per 4×10^8 erythrocytes.

Measurement of cAMP accumulation in human erythrocytes in response to PGI_2 analogs in the absence and presence of PDE inhibitors

Washed erythrocytes diluted to a 50% hematocrit (1 mL) were incubated with either UT-15C (1 μM or 100 nM) or iloprost (100 nM), and after 45 min, the reaction was stopped by the addition of ice-cold ethanol/HCl.[27] Samples were centrifuged at $14{,}000 \times g$ for 10 min at 4°C, and the supernatant was removed and stored overnight at −20°C to precipitate the remaining proteins. The samples were again centrifuged at $3700 \times g$ for 10 min at 4°C. The supernatant was removed and dried under vacuum centrifugation. The dried sample was reconstituted in assay buffer, and the concentration of cAMP was determined using an enzyme immunoassay (GE Healthcare, Piscataway, NJ, USA). Cell counts were obtained from the erythrocyte suspension at a hematocrit of 50%. Levels of cAMP are reported as picomoles per 10^{10} erythrocytes. In separate studies, cAMP levels were determined after UT-15C (100 nM) in the presence of the PDE5 inhibitor, ZAP (10 μM), after iloprost (100 nM) in the presence of the PDE3 inhibitor, CILO (100 μM) or after the vehicle for the PDE inhibitors, DMF.

Measurement of ATP release from erythrocytes in response to low O_2 tension

Washed erythrocytes were diluted to a 20% hematocrit in a Ringers buffer containing bicarbonate (4.7 mM KCl, 2.0 mM $CaCl_2$, 140.5 mM NaCl, 1.2 mM $MgSO_4$, 11.0 mM glucose, 24.8 mM $NaHCO_3$, pH 7.4) at 37°C. Erythrocytes were equilibrated for 20 min in a thin-film tonometer (model 237, Instrumentation Laboratory, Bedford, MA, USA)[28] with a gas mixture containing 15% O_2, 6% CO_2, balance N_2

(mean $pO_2 = 107 \pm 4$ mm Hg, normoxia) in the presence of the PDE5 inhibitor, ZAP or its vehicle (DMF).[28,29] The gas mixture was then changed to one containing 2% O_2, 6% CO_2 and balance N_2 (mean $pO_2 = 18 \pm 1$ mm Hg). Amounts of ATP released from erythrocytes were determined as described above during normoxia as well as following a 10-min exposure of erythrocytes to low pO_2. The pH, pO_2 and pCO_2 were determined during exposure to each gas mixture using a blood gas analyser (model pHOx, Nova Biomedical, Waltham, MA, USA).

Measurement of free hemoglobin

To exclude the presence of significant hemolysis in studies where the release of ATP was measured, samples were centrifuged at $500 \times g$ 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.[29,30] If any increase in free hemoglobin was detected, the studies were not included.

Data analysis

Statistical significance among groups was determined using an analysis of variance (ANOVA). In the event that the F ratio indicated that a change had occurred, a Fisher's LSD test was performed to identify individual differences. Results are reported as mean ± the standard error (SE).

Results

Effect of UT-15C and iloprost on cAMP levels and ATP release from human erythrocytes

Previously, it was shown that activation of the erythrocyte IPR stimulates ATP release via a signalling pathway that requires increases in cAMP.[9] Consistent with this report, we confirmed that UT-15C (1 μM) stimulates increases in cAMP accumulation ($n = 15$, $P < 0.01$, Figure 1a) as well as ATP release from human erythrocytes ($n = 13$, $P < 0.05$, Figure 1b). We next investigated if a 10-fold smaller concentration of PGI_2 analogs would also stimulate cAMP accumulation in these cells. As depicted in Figure 2a, iloprost ($n = 10$) and UT-15C ($n = 10$), at a concentration of 100 nM, each stimulated increases in cAMP levels. Moreover, CILO (100 μM) augmented iloprost-induced increases in cAMP, confirming previous reports that PDE3 regulates cAMP levels in this signalling pathway ($n = 9$, $P < 0.01$, Figure 2b).[22]

Effect of ZAP on UT-15C-induced increases in cAMP levels in human erythrocytes

Although PDE3 hydrolyzes cAMP, it also binds cGMP. The binding of cGMP to the catalytic site of PDE3 inhibits its ability to hydrolyze cAMP.[24] Erythrocytes possess both soluble guanylyl cyclase[31] and PDE5,[23] a PDE that hydrolyzes cGMP.[32] Therefore, we investigated if an inhibitor of PDE5 would augment UT-15C-induced increases in cAMP in human erythrocytes. Erythrocytes were incubated with ZAP (10 μM) or its vehicle (DMF), followed by the addition of UT-15C (100 nM, $n = 6$). This concentration of UT-15C

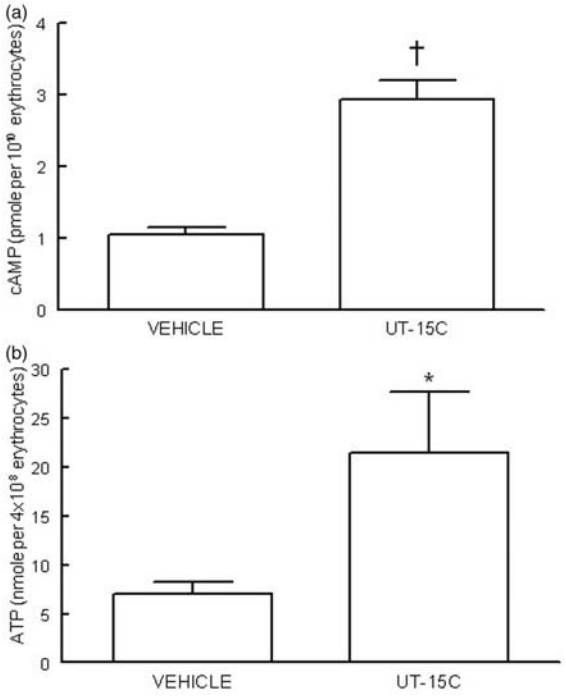

Figure 1 Effect of UT-15C (1 μM) on cAMP levels ($n = 15$, panel a) and ATP release ($n = 13$, panel b) from healthy human erythrocytes. Panel a: † = different from VEHICLE (saline) ($P < 0.01$). Panel b: * = different from VEHICLE (saline) ($P < 0.05$). Values are mean ± SE

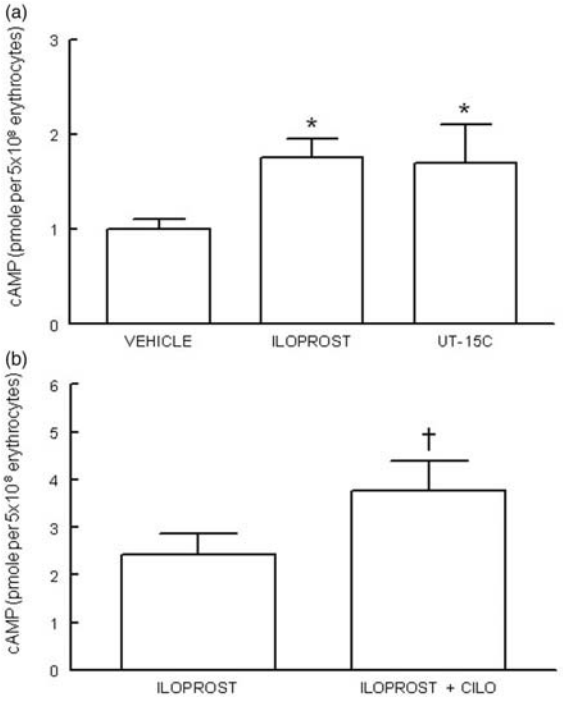

Figure 2 Panel a: Effect of iloprost ($n = 10$) or UT-15C ($n = 10$) at a concentration of 100 nM on cAMP levels in healthy human erythrocytes. * = different from VEHICLE (dimethyl formamide) ($P < 0.05$). Panel b: Effect of preincubation of erythrocytes with cilostazol (CILO, 100 μM) for 30 min prior to the addition of ILOPROST (100 nM, $n = 9$) on cAMP levels. † = different from ILOPROST in the absence of CILO ($P < 0.01$). Values are mean ± SE

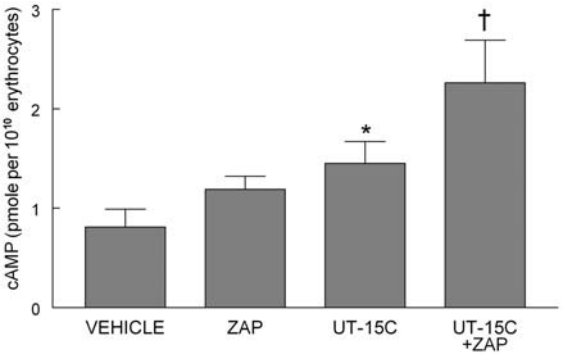

Figure 3 Effect of zaprinast (ZAP, 10 μM) on UT-15C-induced increases in cAMP levels in healthy human erythrocytes. Erythrocytes were incubated with ZAP or its vehicle, dimethyl formamide, for 30 min prior to the addition of UT-15C (100 nM, $n = 6$). Values are the means ± SE. * = different from VEHICLE ($P < 0.05$), † = different from VEHICLE and ZAP ($P < 0.01$) and from UT-15C alone ($P < 0.05$). Values are mean ± SE

stimulated increases in cAMP in the absence of ZAP ($P < 0.05$). However, in the presence ZAP, UT-15C induced greater increases in cAMP ($P < 0.05$, Figure 3).

Effect of PDE5 inhibitors on UT-15C-induced ATP release from human erythrocytes

To determine if the augmented increases in cAMP produced by UT-15C in the presence of ZAP resulted in enhanced ATP release, erythrocytes were incubated for 30 min with one of two chemically dissimilar PDE5 inhibitors, ZAP (10 μM) or TAD (10 μM) or their vehicle (DMF) followed by UT-15C (100 nM, $n = 7$). Incubation of erythrocytes with this concentration of UT-15C in the absence of PDE5 inhibitors did not result in ATP release (Figure 4). However, in the presence of either ZAP (Figure 4a) or TAD (Figure 4b), the same concentration of UT-15C stimulated ATP release ($P < 0.01$).

Effect of ZAP on low-oxygen tension-induced ATP release from human erythrocytes

Erythrocytes also release ATP in response to low oxygen tension (pO_2) via a signalling pathway that differs from the one activated by PGI_2 receptor agonists.[11,33,34] To determine if PDE5 inhibitors also augment ATP release in response to a different physiological stimulus, the effect of ZAP (10 μM) on low-pO_2-induced ATP release was determined. Exposure of erythrocytes to low O_2 tension (18 ± 1 mm Hg) resulted in ATP release that was not altered in the presence of ZAP ($n = 4$, $P < 0.01$, Figure 5). This finding is consistent with the hypothesis that the effect of PDE5 inhibitors on ATP release is localized to the pathway stimulated by PGI_2 receptor agonists.

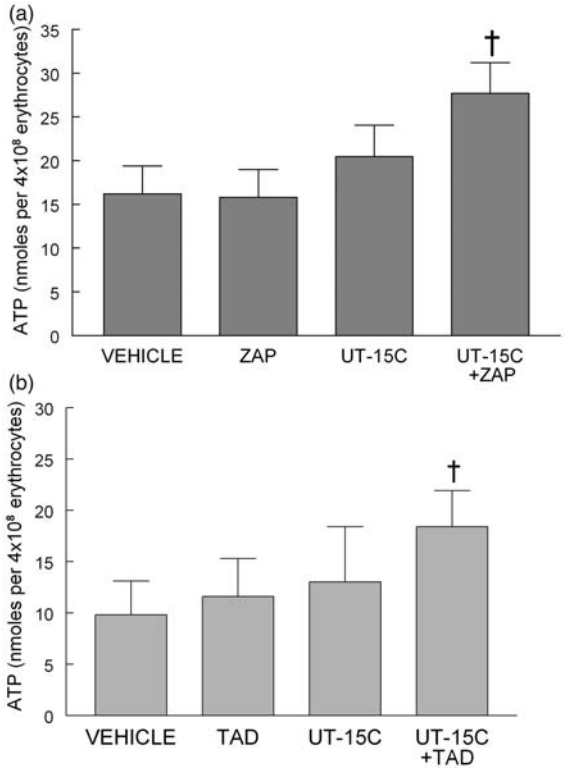

Figure 4 Effect of zaprinast (ZAP, 10 μM, Panel a, $n = 7$) and tadalafil (TAD, 10 μM, $n = 5$) on UT-15C-induced ATP release from healthy human erythrocytes. Erythrocytes were incubated with either ZAP, TAD or their vehicle, dimethyl formamide, for 30 min prior to the addition of UT-15C (100 nM). † = different from VEHICLE and ZAP ($P < 0.01$). Panel b, † = different from all other groups ($P < 0.01$). Values are mean ± SE

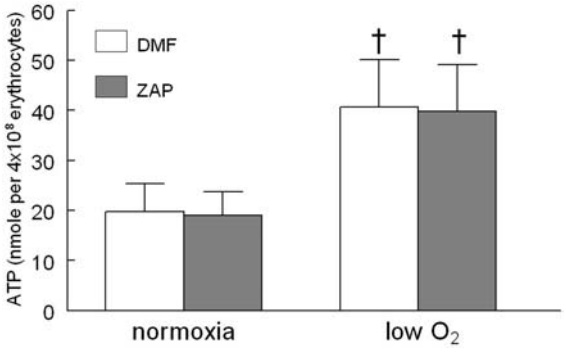

Figure 5 Effect of exposure to low O_2 tension on ATP release from human erythrocytes ($n = 4$). In a tonometer, isolated erythrocytes (hematocrit 20%) were exposed to gas mixtures containing either 15% O_2, 6% CO_2, balance nitrogen (normoxia, $pO_2 = 107 \pm 4$ mmHg) or 2% O_2, 6% CO_2, balance nitrogen (low O_2, $pO_2 = 18 \pm 1$ mmHg). ATP release was determined after a 30-min equilibration with 15% O_2 and 10 min after exposure to 2% O_2. Studies were performed in the presence of zaprinast (ZAP, 10 μM) or its vehicle, dimethyl formamide (DMF). † = different from corresponding normoxia value ($P < 0.01$). Values are the means ± SE

Discussion

The effect of PGI_2 and its active analogs on vascular smooth muscle has been extensively investigated. PGI_2 is released endogenously from the endothelium but can also be administered therapeutically as a sodium salt or an active PGI_2 analog. Once in the circulation, these vasoactive lipids bind to IPRs on platelets, endothelial cells, vascular smooth muscle cells and, importantly, erythrocytes.[7,8,27,35] The cellular responses initiated by activation of the IPR include antiplatelet effects, protection against endothelial stress and relaxation of vascular smooth muscle.[7] More recently, it has been shown that binding of PGI_2 analogs to the

human erythrocyte IPR results in activation of a signalling pathway that results in increases in cAMP and ATP release.[9,27]

Since increases in cAMP are required for IPR-mediated ATP release from erythrocytes, the activity of the pathway-specific PDE(s) that degrade cAMP is critical for the regulation of ATP release.[22,27] At a concentration of 1 μM, the PGI_2 analog, UT-15C, stimulates both increases in cAMP and ATP release from human erythrocytes (Figure 1), a result that is similar to that observed previously for this concentration of iloprost. Here, we demonstrate that a 10-fold smaller concentration of either of these two PGI_2 analogs, iloprost or UT-15C, also stimulates increases in cAMP in human erythrocytes, with iloprost-induced increases in cAMP augmented by the PDE3 inhibitor, cilostazol (Figure 2). The latter finding confirms that PDE3 regulates increases in cAMP associated with activation of this pathway.[22]

Recently, it was shown that PDE5 is present in the cytosolic fraction of human erythrocytes and that the PDE5 inhibitor, ZAP, augments increases in cGMP resulting from activation of soluble guanylyl cyclase in these cells.[23] Since cGMP inhibits the ability of PDE3 to hydrolyze cAMP,[24] we hypothesized that increases in cGMP would augment both cAMP accumulation and ATP release resulting from IPR activation. The major finding of this study is that either of two chemically dissimilar inhibitors of PDE5 augments increases in cAMP produced by incubation of erythrocytes with an IPR agonist, UT-15C (Figure 3). Although UT-15C at a concentration of 100 nM stimulated an increase in cAMP, that increase was potentiated in the presence of the PDE5 inhibitor, ZAP (Figure 3). In spite of the observation that UT-15C at 100 nM stimulated an increase in cAMP in the absence of a PDE5 inhibitor, that increase was not sufficient to stimulate fully the signalling pathway for ATP release (Figure 4). However, when erythrocytes were pretreated with either ZAP (Figure 4a) or TAD (Figure 4b), this concentration of UT-15C did stimulate ATP release from these cells. Although it was reported that PGI_2 analogs and PDE3 inhibitors act synergistically to increase erythrocyte ATP release, these drugs are not generally used in combination in humans. However, PGI_2 analogs and inhibitors of PDE5 are used both individually and in combination with the treatment of PAH.[1,36]

To ascertain if the effects of PDE5 inhibitors on ATP release are specific to the IPR signalling pathway, we determined the effects of ZAP on ATP release in response to exposure of erythrocytes to low oxygen (O_2) tension that activates a signalling pathway for ATP release that is distinct from that of the IPR pathway.[11,33,34] Here, we determined that under conditions in which ZAP augmented IPR agonist-induced ATP release, the PDE5 inhibitor had no effect on low O_2-induced ATP release (Figure 5). This finding is consistent with the hypothesis that PDE5 activity is not associated with all signalling pathways for ATP release from human erythrocytes.

In summary, our results demonstrate a role for both PDE3 and PDE5 in the regulation of IPR-mediated increases in cAMP and ATP release from human erythrocytes. The proposed relationships among IPR activation, PDE3 and PDE5 in these cells are depicted in Figure 6. These findings have implications for the development of new therapeutic approaches to the treatment of conditions such as PAH. Although PGI_2 analogs are particularly effective in the treatment of PAH, adverse effects including gastrointestinal distress, headache and hypotension can limit the dose of PGI_2 analog that can be administered.[37–39] The finding that PDE5 inhibitors potentiate the UT-15C-induced ATP release from human erythrocytes suggests that, when combined with a PDE5 inhibitor, the concentration of PGI_2 analog required to lower pulmonary arterial pressure in humans with PAH could be reduced resulting in a decrease in unwanted side effects.

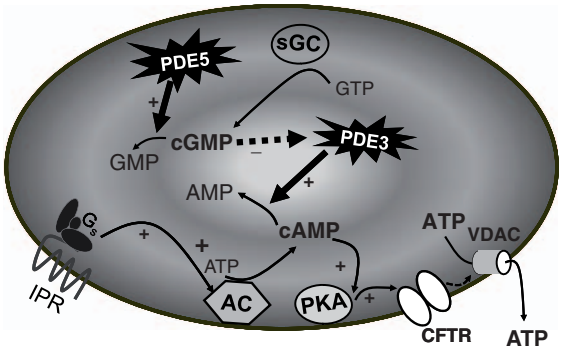

Figure 6 Proposed model of the mechanism by which phosphodiesterase 3 (PDE3) and PDE5 inhibitors potentiate prostacyclin (PGI_2) analog-induced stimulation of increases in cAMP and ATP release from human erythrocytes. Abbreviations: AC: adenylyl cyclase; AMP: adenosine monophosphate; ATP: adenosine 5′ triphosphate; cAMP: 3′,5′-cyclic adenosine monophosphate; CFTR: cystic fibrosis transmembrane conductance regulator; cGMP: 3′,5′-cyclic guanosine monophosphate; GMP: guanosine monophosphate; GTP: guanosine triphosphate; Gs: the heterotrimeric G protein, Gs; IPR: prostacyclin receptor; PDE3: phosphodiesterase 3; PDE5: phosphodiesterase 5; PKA: protein kinase A; sGC: soluble guanylyl cyclase; VDAC: voltage-dependent anion channel; (+): activation; (−): inhibition

Author contributions: All authors reviewed the manuscript and performed data analysis. SMK, EAB, MLE, RSS and AHS participated in research design. SMK, MME, EAB, AKZ and RSS conducted experiments. SMK, EAB, AHS, MLE and RSS wrote or contributed to the writing of the manuscript.

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