

Diamide decreases deformability of rabbit erythrocytes and attenuates low oxygen tension-induced ATP release

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Abstract

Exposure of erythrocytes to reduced oxygen (O_2) tension activates the heterotrimeric G-protein G_i , resulting in the accumulation of cyclic AMP (cAMP) and release of ATP. The mechanism by which exposure of erythrocytes to reduced O_2 tension activates G_i is not known. Here we investigate the hypothesis that, in rabbit erythrocytes, ATP release in response to exposure to reduced O_2 tension is linked to erythrocyte membrane deformability. If this hypothesis is correct, then decreasing the deformability of the erythrocyte membrane should decrease the release of ATP in response to reduced O_2 tension. We report that treating erythrocytes with diamide, a compound that decreases erythrocyte deformability, inhibits low O_2 tension-induced ATP release. Treating erythrocytes with diamide does not, however, interfere with cAMP accumulation or ATP release in response to a direct activator of G_i (mastoparan 7) or in response to receptor-mediated activation of G_s (the prostacyclin analog, iloprost). These results demonstrate that diamide (100 $\mu\text{mol/L}$) does not directly inhibit the signaling pathways for ATP release from rabbit erythrocytes and support the hypothesis that low O_2 tension-induced ATP release from these cells is linked to membrane deformability.

Keywords: red blood cell, diamide, cAMP, mastoparan 7, iloprost

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Introduction

The mammalian erythrocyte, the anucleated cell primarily responsible for transporting oxygen (O_2), has also been proposed to regulate local vascular resistance in the microcirculation via its ability to release ATP when exposed to reduced O_2 tension.^{1,2} This erythrocyte-derived ATP can then bind to purinergic receptors found on endothelial cells leading to a vasodilation that is conducted to upstream feed arterioles.^{3–5} Thus, the release of ATP from erythrocytes in response to local decreases in O_2 tension provides a mechanism by which the cell responsible for O_2 delivery can increase blood flow to areas requiring increased O_2 supply.¹

The release of ATP resulting from exposure of erythrocytes to low O_2 tension requires activation of the heterotrimeric G protein G_i .^{6,7} In addition to exposure to low O_2 tension, subjecting erythrocytes to mechanical deformation also leads to the activation of G_i and subsequent ATP release.^{7–9} The activation of G_i initiates a signal transduction pathway that includes adenylyl cyclase and requires increases in cyclic AMP (cAMP) for ATP release to occur.^{6,7,9,10} Previously, G_i has been shown to be activated by shear stress applied to both epithelial and endothelial

cells.^{11,12} Therefore, the finding that mechanical deformation activates G_i in the erythrocyte membrane is inherently consistent with previous findings. However, the mechanism by which the exposure of the erythrocyte to low O_2 tension activates G_i -mediated signaling has not been elucidated.

Hemoglobin, the primary O_2 carrier within the erythrocyte, is a tetrameric protein that changes conformation based on its oxygenation status. It was reported previously that in severe hypoxia, O_2 content of blood is more important than O_2 tension in the maintenance of O_2 supply in skeletal muscle.^{4,13} Since the O_2 saturation of hemoglobin determines the O_2 content of blood, this would implicate hemoglobin in the ATP release signal transduction pathway. Indeed, it has been shown that ATP release from erythrocytes is dependent on the O_2 saturation of the hemoglobin molecules rather than the O_2 tension to which the cell is exposed.¹⁴

Here we investigate the hypothesis that, in rabbit erythrocytes, ATP release in response to exposure to reduced O_2 tension is linked to erythrocyte membrane deformability. Under this hypothesis, decreasing the deformability of the

erythrocyte membrane would decrease the release of ATP in response to reduced O₂ tension. We used diamide, a compound that cross-links spectrin molecules, to reduce erythrocyte membrane deformability¹⁵ and evaluated ATP release in response to exposure to lowered O₂ tension. In addition, we determined the effect of diamide on ATP release in response to exposure of erythrocytes to direct stimulation of Gi by mastoparan 7 (MAS7) as well as in response to receptor-mediated activation of Gs by the prostacyclin analog, iloprost (ILO). Although diamide-treated erythrocytes release ATP in response to both direct activation of Gi and receptor-mediated activation of Gs, ATP release in response to exposure to reduced O₂ tension was significantly attenuated following diamide incubation. These results support the hypothesis that deformability of the erythrocyte membrane is linked to low O₂ tension-induced ATP release.

Methods

Isolation of rabbit erythrocytes

Male New Zealand white rabbits were anesthetized with ketamine (12.5 mg/kg) and xylazine (1.5 mg/kg) intramuscularly, followed by pentobarbital sodium (10 mg/kg) administered via a cannula placed in an ear vein. A catheter was placed in a carotid artery, heparin (500 U) was administered, and after 10 min the animals were exsanguinated. Immediately after collection, the whole blood was centrifuged at 500 *g* at 4°C for 10 min and the plasma, buffy coat and uppermost erythrocytes were removed by aspiration and discarded. The remaining erythrocytes were washed three times in buffer containing 21.0 mmol/L tris(hydroxymethyl)aminomethane, 4.7 mmol/L KCl, 2.0 mmol/L CaCl₂, 140.5 mmol/L NaCl, 1.2 mmol/L MgSO₄, 5.5 mmol/L glucose and 0.5% bovine albumin fraction V, final pH 7.4. Erythrocytes isolated in this manner contain fewer than one leukocyte per 50 high power fields (approximately 8–10 leukocytes per mm³) and are devoid of platelets.¹⁶ Cells were prepared on the day of use.

Measurement of erythrocyte deformability

Isolated erythrocytes, exposed to room air, were diluted in the above buffer to a hematocrit of 10% and were incubated with diamide (100 μmol/L; Sigma-Aldrich, St Louis, MO, USA) or its vehicle (saline). Erythrocyte deformability was measured 30, 45 and 60 min after the addition of diamide or its vehicle. Erythrocyte deformability was assessed using the St George's Blood Filtrationmeter (Carri-Med Ltd, Dorking, UK).^{17,18} This device develops a pressure gradient distal to a vertically mounted 13 mm diameter polycarbonate filter (Nucleopore). The filter has a 9.53 mm exposed surface diameter, filter thickness of 11 μm and average pore size of 5 μm. Proximal to the filter, the chamber and an open-ended capillary tube were filled with either buffer or buffer containing erythrocytes (hematocrit 10%). Upon opening of a tap on the outflow tube, the buffer or buffer containing erythrocytes was subjected to a negative pressure. The fluid was pulled through the filter with the

rate of filtration determined by the time required for the air–fluid meniscus to pass fiberoptic sensors surrounding the capillary tube. The filtration rates for the erythrocyte suspension were determined. Red cell transit time (RCTT), a dimensionless number determined by comparing the filtration rates for the erythrocyte suspension with the filtration rate of buffer alone, is calculated using manufacturer-supplied software and a dedicated computer. An increase in RCTT reflects a decrease in erythrocyte deformability.^{17,18}

Determination of ATP release from erythrocytes in response to exposure to reduced O₂ tension in the absence and presence of diamide

Washed erythrocytes were diluted to a 10% hematocrit in a Ringer's buffer containing bicarbonate (4.7 KCl, 2.0 mmol/L CaCl₂, 140.5 mmol/L NaCl, 1.2 mmol/L MgSO₄, 11 mmol/L glucose, 23.8 mmol/L NaHCO₃, 0.2% dextrose, 0.5% bovine serum albumin, pH 7.4) at 37°C. In the presence of 100 μmol/L diamide or its vehicle (saline), erythrocytes were equilibrated for 30 min in a tonometer (model 237; Instrumentation Laboratory, Bedford, MA, USA) with a gas mixture containing 15% O₂, 6% CO₂, balance N₂ (normoxia, *p*O₂ = 117.0 ± 4.1 mmHg). The incubation time of erythrocytes with diamide was chosen based on the preliminary studies demonstrating that diamide significantly increased RCTT after 30 min. The gas mixture was then changed to one containing 4.5% O₂, 6% CO₂, balance N₂ to achieve a *p*O₂ of 35.0 ± 1.9 mmHg. Finally, the gas mixture was changed to one containing 0% O₂, 6% CO₂, balance N₂ to achieve a *p*O₂ of 10.9 ± 1.3 mmHg. The pH, *p*O₂ and *p*CO₂ of the red blood cell suspension were determined after a 10-min exposure to each gas mixture (*p*H₀; Nova Biomedical, Waltham, MA, USA) (Table 1). The amount of ATP released from erythrocytes was determined during normoxia and following the 10-min exposure to each gas mixture and is reported as a percent change from normoxia. Although the tonometer spins the erythrocyte suspension, the shear stress placed on the erythrocytes in this device is minimal and is not sufficient to stimulate ATP

Table 1 pH, *p*CO₂, *p*O₂ (*n* = 8) and SO₂ (*n* = 3) of saline- and diamide- (100 μmol/L)-treated erythrocytes

	Saline-treated erythrocytes	Diamide-treated erythrocytes
15% O₂, 6% CO₂		
pH	7.43 ± 0.01	7.42 ± 0.01
<i>p</i> CO ₂	36.7 ± 1.0	37.3 ± 0.7
<i>p</i> O ₂	116.1 ± 5.7	118.0 ± 6.2
SO ₂	96.0 ± 0.3	97.1 ± 1.7
4.5% O₂, 6% CO₂		
pH	7.42 ± 0.01	7.42 ± 0.01
<i>p</i> CO ₂	38.3 ± 0.6	38.3 ± 0.3
<i>p</i> O ₂	35.4 ± 2.84	34.5 ± 2.82
SO ₂	49.0 ± 1.5	50.4 ± 1.6
0% O₂, 6% CO₂		
pH	7.43 ± 0.01	7.44 ± 0.01
<i>p</i> CO ₂	38.0 ± 0.6	38.3 ± 0.4
<i>p</i> O ₂	11.1 ± 1.8	10.8 ± 2.0
SO ₂	13.7 ± 0.6	13.7 ± 2.7

release. Baseline ATP levels in suspensions of erythrocytes exposed to normoxia in the tonometer or placed in a test tube at the same hematocrit were 7.4 ± 2.7 ($n = 8$) and 9.6 ± 2.3 ($n = 15$) nanomoles per 4×10^8 erythrocytes, respectively.

In separate experiments, hemoglobin O₂ saturations (OSM3, Radiometer) were determined following exposure of the erythrocytes to the three different gas mixtures in the presence and absence of diamide.

Incubation of erythrocytes with pharmacological agents that stimulate increases in ATP in the absence and presence of diamide

Isolated erythrocytes exposed to room air were diluted to a hematocrit of 10% in buffer containing 21.0 mmol/L tris(hydroxymethyl)aminomethane, 4.7 mmol/L KCl, 2.0 mmol/L CaCl₂, 140.5 mmol/L NaCl, 1.2 mmol/L MgSO₄, 5.5 mmol/L glucose and 0.5% bovine albumin fraction V, final pH 7.4. Erythrocytes were then preincubated with diamide (100 μ mol/L) or its vehicle (saline) for 30 min followed by either the direct activator of Gi, MAS7 (10 μ mol/L; GenScript, Piscataway, NJ, USA) or the prostacyclin analog, ILO (1 μ mol/L; Cayman, Ann Arbor, MI, USA), an activator of the Gs-coupled prostacyclin receptor. ATP release was measured after 5, 10 and 15 min. Peak ATP values are reported.

Measurement of ATP

ATP was measured using the luciferin-luciferase assay as described previously.^{18,19} A 200 μ L sample of erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 100 μ L of firefly lantern extract (10 mg/mL, FLE 250; Sigma) and 100 μ L of a solution of synthetic D-luciferin (50 mg/100 mL; Sigma). The light emitted was detected using a luminometer (Turner Designs, Sunnyvale, CA, USA). A standard curve for ATP (Sigma) was obtained for each experiment. Cell counts were obtained from the suspension of erythrocytes and amounts of ATP measured were normalized to 4×10^8 cells.

Measurement of total intracellular ATP of erythrocytes

A known number of erythrocytes, determined by counting the number of erythrocytes in a known volume using a hemocytometer, were lysed in distilled water. ATP was measured as described above. Values were normalized to ATP concentration per erythrocyte.

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 *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. If increases in free hemoglobin were detected, the data were excluded to ensure that levels of ATP reported do not reflect that released as a result of erythrocyte lysis.

Incubation of erythrocytes with pharmacological agents that stimulate increases in cAMP in the absence and presence of diamide

Isolated erythrocytes exposed to room air were diluted to a 50% hematocrit in buffer containing 21.0 mmol/L tris(hydroxymethyl)aminomethane, 4.7 mmol/L KCl, 2.0 mmol/L CaCl₂, 140.5 mmol/L NaCl, 1.2 mmol/L MgSO₄, 5.5 mmol/L glucose and 0.5% bovine albumin fraction V, final pH 7.4. Erythrocytes were then incubated with diamide (100 μ mol/L) or its vehicle (saline) for 30 min. The erythrocytes were subsequently incubated with either MAS7 (30 μ mol/L, 30 min) or ILO (1 μ mol/L, 15 min). Reactions were stopped with the addition of 4 mL of ice-cold ethanol containing 1 mmol/L HCl. The incubation times and agonist concentrations were chosen based on preliminary studies.^{6,20}

Measurement of cAMP

The erythrocyte-ethanol mixture was centrifuged at 14,000 *g* for 10 min at 4°C to remove precipitated proteins. The supernatant was removed and stored overnight at -20°C. Samples were centrifuged a second time at 3700 *g* for 10 min at 4°C to remove cryoprecipitates. The supernatant was removed and dried under vacuum centrifugation. Concentrations of cAMP were determined by enzyme immunoassay (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions.

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 least significant difference test was performed to identify individual differences. When appropriate a Student's *t*-test was used. Results are reported as means $\pm$ standard error (SE).

Institutional approval

The protocol describing the use of rabbits was approved by the Institutional Animal Care and Use Committee of Saint Louis University.

Results

Effect of diamide on erythrocyte deformability

The exposure of erythrocytes to 100 μ mol/L diamide for 30 min resulted in a decrease in erythrocyte deformability (increase in RCTT) that persisted for 30 additional min (Figure 1; $n = 4$). Importantly, this concentration of diamide had no effect on basal cAMP concentration or total ATP content of erythrocytes (Table 2).

Effect of diamide on low O₂ tension-induced ATP release from rabbit erythrocytes

Baseline ATP release (incubation with 15% O₂/6% CO₂) between saline- and diamide-treated cells was not different (7.4 ± 3.6 and 10.1 ± 2.9 nmol per 4×10^8 erythrocytes, respectively). However, erythrocytes pretreated with diamide released significantly less ATP in response to reduced O₂

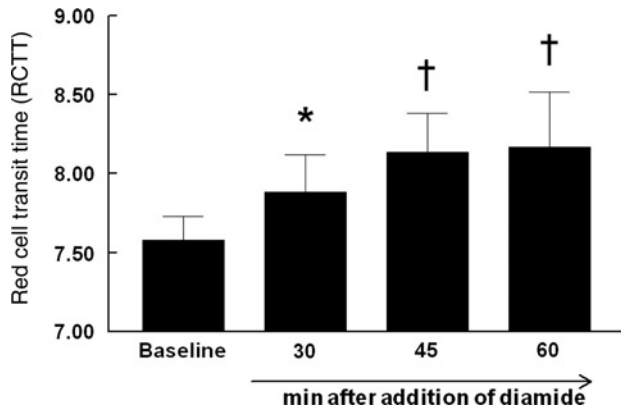


Figure 1 Effect of diamide on red cell transit time (RCTT). Rabbit erythrocytes were incubated with 100 $\mu\text{mol/L}$ diamide for 30, 45 and 60 min and RCTT was measured ($n = 4$). Values are the means $\pm$ SE. * $P < 0.05$, different from baseline; † $P < 0.01$, different from baseline

tension than erythrocytes pretreated with the vehicle for diamide (saline) (Figure 2; $n = 8$). The $p\text{CO}_2$, pH, $p\text{O}_2$ and SO_2 of the red blood cell suspension achieved at each gas composition were not significantly different between the diamide- and saline-treated groups (Table 1), demonstrating that O₂ affinity of hemoglobin was not changed by diamide.

Effect of diamide on the ability of erythrocytes to accumulate cAMP and release ATP in response to activation of Gi

To establish that the Gi-mediated pathway for ATP release from erythrocytes that is activated in response to lowered O₂ tension is intact in diamide-treated erythrocytes, cAMP levels and ATP release in response to MAS7 were measured in the absence and presence of diamide. Baseline ATP release between diamide- and saline-treated cells was not different (12.7 ± 3.6 and 14.4 ± 3.7 nmol per 4×10^8 erythrocytes, respectively). Diamide (100 $\mu\text{mol/L}$, 30 min) also did not alter increases in either cAMP (Figure 3a; $n = 5$) or ATP release (Figure 3b; $n = 8$) in response to incubation of erythrocytes with 30 or 10 $\mu\text{mol/L}$ MAS7, respectively. These results indicate that the previously described signal transduction pathway for ATP release in response to reduced O₂ tension⁷ is intact in diamide-treated erythrocytes.

Effect of 100 $\mu\text{mol/L}$ diamide on ILO-induced cAMP accumulation and ATP release

To ensure that erythrocytes treated with diamide remained capable of accumulating cAMP and releasing ATP in response to receptor-mediated activation of Gs, a stimulus for ATP release that is independent of changes in

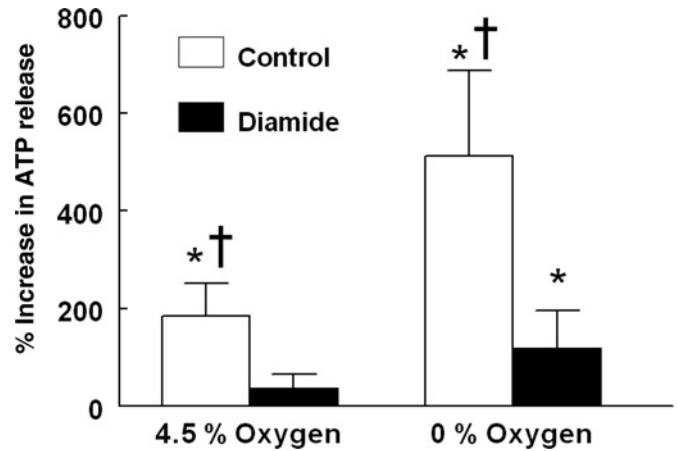


Figure 2 Effect of diamide on low O₂ tension-induced ATP release from rabbit erythrocytes. Erythrocytes were incubated with 100 $\mu\text{mol/L}$ diamide or its vehicle (saline) for 30 min while exposed to gas with a composition of 15% O₂, 6% CO₂, balance N₂ in a tonometer. The cells were then exposed sequentially to gases with compositions of 4.5% O₂, 6% CO₂, balance N₂ and 0% O₂, 6% CO₂, balance N₂. ATP was measured 30 min after exposure to 15% O₂ and 10 min after exposure to 4.5% O₂ and 0% O₂ ($n = 8$). The percent increase in ATP release from baseline (15% O₂) is reported. pH, $p\text{O}_2$, $p\text{CO}_2$ and SO_2 are reported in Table 1. Values are the means $\pm$ SE. * $P < 0.05$, different from respective baseline; † $P < 0.05$, different from respective diamide-treated group

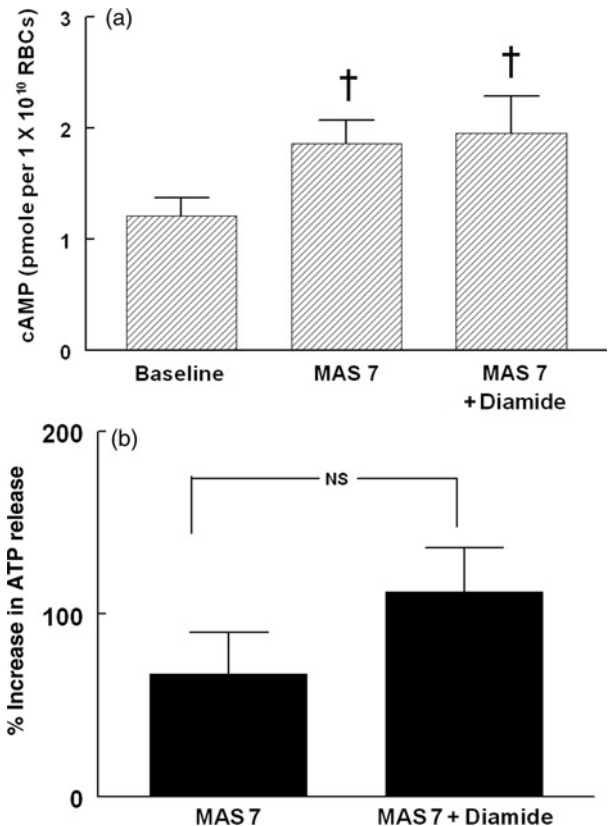


Figure 3 Effect of diamide on mastoparan 7 (MAS7)-induced cyclic AMP (cAMP) accumulation (a) and ATP release (b) from rabbit erythrocytes. Erythrocytes were incubated with 100 $\mu\text{mol/L}$ diamide or its vehicle (saline) 30 min before the addition of MAS7. cAMP accumulation (a) was measured 30 min after the addition of MAS7 (30 $\mu\text{mol/L}$, $n = 5$) and ATP release (b) was measured 5, 10 and 15 min after the addition of MAS7 (10 $\mu\text{mol/L}$, $n = 8$). For ATP release, percent increase from baseline for peak values (occurring at 9.7 ± 0.9 min after treatment with MAS7) is reported. Values are the means $\pm$ SE. † $P < 0.01$, different from baseline; NS, not significantly different

Table 2 Effect of diamide (100 $\mu\text{mol/L}$) on baseline cAMP levels ($n = 8$) and intracellular ATP levels ($n = 21$) in rabbit erythrocytes

Group	cAMP (pmol per 10^{10} RBCs)	ATP (mmol/L)
Saline treated	0.47 ± 0.054	2.53 ± 0.225
Diamide treated	0.53 ± 0.049	2.54 ± 0.235

cAMP, cyclic AMP; RBC, red blood cells
Values are means $\pm$ SE

deformability, rabbit erythrocytes were incubated with the prostacyclin analog, ILO (1 $\mu\text{mol/L}$) in the absence and presence of diamide. Diamide pretreatment (100 $\mu\text{mol/L}$, 30 min) had no effect on baseline ATP levels (6.0 ± 2.3 and 8.0 ± 3.7 nmol per 4×10^8 erythrocytes in the absence and presence of diamide, respectively) and did not alter ILO-induced increases in cAMP (Figure 4a; $n = 9$) or ATP release (Figure 4b; $n = 7$).

Discussion

Erythrocytes release ATP in response to exposure to reduced O_2 tension as would be encountered as these cells traverse the microcirculation of metabolically active tissue.^{1,2,5} The ability of the erythrocyte to release ATP in response to lowered O_2 tension has been suggested to permit this cell to participate in the matching of O_2 supply with demand in skeletal muscle.^{1,4} It has been demonstrated that ATP

release in response to either mechanical deformation or exposure to reduced O_2 tension requires activation of the heterotrimeric G protein, Gi .^{6,7} In addition to the latter stimuli, human and rabbit erythrocytes release ATP in response to agonist-induced activation of β -adrenergic receptors and prostacyclin receptors (IPR), both coupled to the heterotrimeric G protein, Gs .^{20,21}

Regardless of whether Gi or Gs is activated in erythrocytes, signaling pathways are initiated that include adenylyl cyclase and protein kinase A.^{6,9,10,22} Although the contribution of the individual components of these signaling pathways to ATP release have been studied, the mechanism by which Gi is activated in the erythrocyte in response to lowered O_2 tension was not established.

Here we determined that diamide, at a concentration which reduced erythrocyte deformability (100 $\mu\text{mol/L}$), selectively attenuates ATP release in response to exposure of erythrocytes to reduced O_2 tension. Importantly, the concentration of diamide used had no effect on total intracellular ATP levels or on ATP release in response to either direct activation of Gi or receptor-mediated activation of the IPR. These results are consistent with the hypothesis that low O_2 tension-induced ATP release from rabbit erythrocytes is linked to membrane deformability.

Several studies in endothelial cells and other cell types have demonstrated that G proteins, and in particular, Gi , can be activated through mechano-transduction (shear stress).^{11,12} The erythrocyte membrane-cytoskeleton is a complex network of interconnecting proteins. The major extrinsic proteins of the cytoskeleton are spectrin (bands 1 and 2), ankyrin (band 2.1), band 4.1 and actin (band 5). Of these proteins, spectrin accounts for 75% of the mass of the cytoskeleton and forms a mesh-like network that is attached to the erythrocyte membrane through ankyrin. Ankyrin is bound to the membrane through interactions with the cytoplasmic domain of the integral membrane protein band 3. The spectrin-ankyrin-band 3 interaction provides the major association between the cytoskeleton and erythrocyte membrane.²³ Although the majority of hemoglobin is present in the cytoplasm of erythrocytes, hemoglobin is also found associated with the cytoplasmic surface of the erythrocyte membrane where it interacts with membrane proteins, including band 3.²⁴ Studies by Jagger *et al.*¹⁴ demonstrated that the amount of ATP released from the erythrocyte during exposure to reduced O_2 tension is highly correlated with the hemoglobin O_2 saturation. In the same study, it was demonstrated that the conformational change of hemoglobin from its 'oxygenated' to 'deoxygenated' state must occur for ATP to be released when erythrocytes are exposed to lowered O_2 tension.¹⁴ Although this study demonstrated that decreases in hemoglobin saturation serve as the signal for ATP release, the mechanism that relates these decreases with a signaling pathway that regulates ATP release was not established. One mechanism that could couple reductions in hemoglobin saturation with ATP release is if the conformational changes that occur upon hemoglobin desaturation induce local membrane deformations. These local membrane deformations could then activate Gi leading to stimulation of the signaling pathway for ATP release.

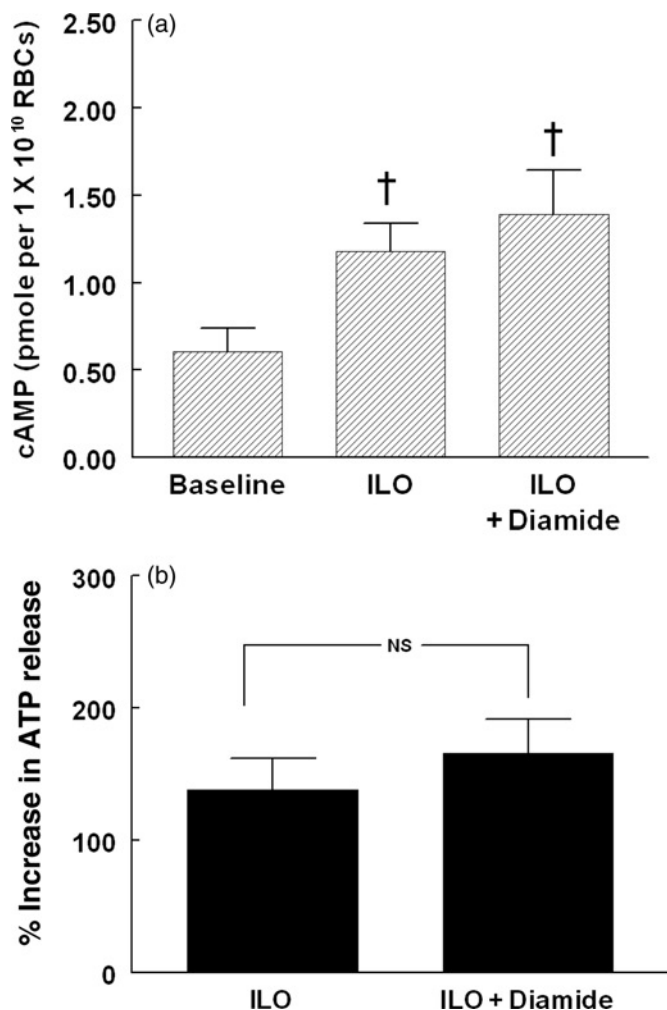


Figure 4 Effect of diamide on iloprost (ILO)-induced cyclic AMP (cAMP) accumulation (a) and ATP release (b) from rabbit erythrocytes. Erythrocytes were incubated with 100 $\mu\text{mol/L}$ diamide or its vehicle (saline) 30 min before the addition of ILO. cAMP accumulation (a) was measured 15 min after the addition of ILO (1 $\mu\text{mol/L}$, $n = 9$) and ATP release (b) was measured after 5, 10 and 15 (1 $\mu\text{mol/L}$, $n = 7$). For ATP release, percent increase from baseline for peak values (occurring at 10.4 ± 1.3 min after treatment with ILO) is reported. Values are the means $\pm$ SE. † $P < 0.01$, different from baseline; NS, not significantly different

The physical properties of the cytoskeletal protein spectrin are essential in maintaining the shape and deformability of the erythrocyte.¹⁵ Diamide, a compound that depletes erythrocyte glutathione levels and oxidizes sulfhydryl groups in spectrin, induces cross-linking of spectrin, producing decreased membrane deformability.^{15,25,26} Importantly, when compared with other compounds used to decrease erythrocyte deformability, diamide has fewer adverse effects on the erythrocyte membrane.^{15,25} At concentrations below 500 $\mu\text{mol/L}$, diamide-induced decreases in erythrocyte deformability can be attributed solely to effects on the viscoelastic properties of the erythrocyte membrane. Thus, in our study, diamide-induced decreases in deformability cannot be attributed to effects on intracellular viscosity or cell shape.^{15,25,27}

It has been previously reported that treating erythrocytes with diamide inhibits ATP release in response to external shear force.^{28,29} In addition, work by Faris and Spence²⁸ demonstrated that exposure to diamide attenuated ATP release from rabbit erythrocytes in response to reduced O₂ tension. However, in the latter study the concentration of diamide used (40 $\mu\text{mol/L}$ for 18 min) was not demonstrated to decrease erythrocyte deformability. In addition, this study did not investigate the effects of diamide on Gi or Gs signaling pathways for ATP release from erythrocytes.

Since diamide is an oxidizing agent, we wished to establish that this agent did not directly inhibit either Gi- or Gs-associated signaling pathways for ATP release from erythrocytes or the ability of these cells to synthesize ATP. In thyroid cells and pancreatic islets, diamide has been demonstrated to either potentiate or inhibit cAMP accumulation depending on the concentration used.^{30,31} Therefore, we measured increases in cAMP and ATP release in response to direct activation of Gi with MAS7- and receptor-mediated activation of Gs with ILO in the absence and presence of diamide. We found that, similar to the effect of diamide on cAMP accumulation in other cell types, high concentrations (5 mmol/L) inhibited ILO-induced ATP release (data not shown). However, as shown in Figures 3 and 4, at 100 $\mu\text{mol/L}$, a concentration that did not alter either basal cAMP or ATP levels, diamide had no effect on MAS7- or ILO-induced increases in cAMP accumulation and ATP release. These results demonstrate that the concentration of diamide used in our studies does not directly inhibit these signaling pathways for ATP release. This is of importance since it has been shown that ATP release in response to exposure of erythrocytes to low O₂ tension requires activation of Gi.⁷

Another potential concern with diamide is that treatment of mouse erythrocytes with diamide at concentrations of 2 mmol/L or greater was reported to induce a loss of cooperativity in the binding of O₂ by hemoglobin.³² In another study using human erythrocytes, it was shown that there was a decrease in P₅₀ (O₂ tension at which hemoglobin is 50% saturated) when erythrocytes were treated with 5 mmol/L diamide, but no change in P₅₀ was observed when erythrocytes were treated with 2 mmol/L diamide.¹⁵ To ensure that the far smaller concentration of diamide used in these studies (100 $\mu\text{mol/L}$) did not alter the ability of hemoglobin to bind or release O₂, hemoglobin, O₂

saturations in the presence and absence of diamide were determined. In rabbit erythrocytes, we observed no differences in the measured hemoglobin O₂ saturations in response to 100 $\mu\text{mol/L}$ diamide (Table 1), demonstrating that the concentration of diamide used in our studies has no effect on the O₂ binding characteristics of hemoglobin.

Taken together, these results are consistent with the hypothesis that diamide inhibits low O₂ tension-induced ATP release by decreasing membrane deformability. The studies described here do not define how hemoglobin interacts with the membrane to produce local deformation when O₂ is released. As noted above, hemoglobin can interact with the membrane by binding to band 3. Previous studies using mouse and human erythrocytes demonstrated that upon binding of hemoglobin to band 3, glycolytic enzymes are disassociated from the membrane and glycolysis is stimulated.^{33,34} In a study using rat erythrocytes, it was suggested that the binding of hemoglobin to band 3 also stimulates ATP release.¹⁴ Thus, it is possible that band 3 is the protein with which hemoglobin interacts to produce local membrane deformation. However, the resolution of this issue is beyond the scope of the current study. It should be noted that treating erythrocytes with diamide has been shown to tyrosine phosphorylate band 3 at the same site that hemoglobin and glycolytic enzymes are known to bind.³⁵ Phosphorylated band 3 is known to have a decreased affinity for hemoglobin and glycolytic enzymes.³⁶ The concentration of diamide used in these studies (100 $\mu\text{mol/L}$) is well below the concentration (300 $\mu\text{mol/L}$) that is required to stimulate tyrosine phosphorylation of band 3, making it unlikely that this effect of diamide is responsible for inhibition of low O₂-induced ATP release.³⁵

In summary, we demonstrated that treating rabbit erythrocytes with diamide (100 $\mu\text{mol/L}$) results in reduced erythrocyte deformability and attenuated ATP release in response to exposure of these cells to reduced O₂ tension. However, this concentration of diamide does not inhibit cAMP accumulation or ATP release in response to receptor-mediated activation of Gs (ILO) or direct activation of Gi (MAS7). These results are consistent with the hypothesis that low O₂ tension-induced ATP release from rabbit erythrocytes is initiated by stress-induced alterations in the cell membrane, resulting in the activation of Gi and that this may be a consequence of the conformational change induced by the desaturation of membrane-associated hemoglobin.

Author contributions: All authors contributed equally to this work.

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