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Insulin inhibits human erythrocyte cAMP accumulation and ATP release: role of PDE3 and PI3K

Madelyn S. Hanson¹, Alan H. Stephenson¹, Elizabeth A. Bowles¹, and Randy S. Sprague¹

¹Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, Missouri

Abstract

In non – erythroid cells, insulin stimulates a signal transduction pathway that results in the activation of phosphoinositide 3 – kinase (PI3K) and phosphorylation of phosphodiesterase 3 (PDE3). Erythrocytes possess insulin receptors, PI3K, and PDE3B. These cells release ATP via a signaling pathway that requires activation of the G protein, Gi, as well as increases in cAMP. Although insulin inhibits ATP release from human erythrocytes in response to Gi activation with mastoparan 7 (Mas 7), no effect on cAMP was described. Here, we investigated the hypothesis that insulin activates PDE3 in human erythrocytes via a PI3K – mediated mechanism resulting in cAMP hydrolysis and inhibition of ATP release. We show that insulin attenuates Mas 7 – induced increases in cAMP and that selective inhibitors of PDE3 (cilostazol) or PI3K (LY294002) rescue this effect of insulin. In addition, we demonstrated that both cilostazol and LY294002 prevent insulin – induced attenuation of Mas 7 – induced ATP release. These results provide support for the hypothesis that insulin activates PDE3 in erythrocytes via a PI3K – dependent mechanism. Once activated, PDE3 limits Mas 7 – induced increases in intracellular cAMP. This effect of insulin leads, ultimately, to decreased ATP release in response to Mas 7. Since the activation of Gi is required for reduced O₂ tension – induced ATP release from erythrocytes, and insulin has been shown to inhibit that release, these results suggest a novel mechanism by which supraphysiological levels of plasma insulin, such as those reported in humans with prediabetes, could inhibit ATP release from erythrocytes. Erythrocyte – derived ATP has been shown to participate in the matching of O₂ supply with demand in skeletal muscle. Thus, pathological increases in circulating insulin could, via activation of PDE3, inhibit ATP release from erythrocytes depriving the peripheral circulation of a mechanism that regulates delivery of O₂ to meet tissue metabolic need.

Keywords

RBC; cilostazol; LY294002; mastoparan 7

Introduction

There is strong evidence that the vascular disease of type II diabetes arises before pathological increases in blood glucose occur (1–3). Indeed, microvascular dysfunction in prediabetes can be as pronounced as that in established type II diabetes. While insulin resistance is present in both prediabetes and type II diabetes, there are marked differences between these two disease states. In type II diabetes, hyperglycemia develops when insulin secretion by the pancreatic β cells is no longer sufficient to compensate for insulin resistance. This decrement in insulin

Address correspondence to: Madelyn S. Hanson, Dept. of Biophysics, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226, stumpfms@slu.edu.

release results in inadequate glucose transport into cells. In contrast, individuals with prediabetes have sufficient β cell function and maintain near normal blood glucose levels by secreting high concentrations of insulin to overcome peripheral insulin resistance resulting in severe hyperinsulinemia (2,4,5). In individuals with prediabetes, it was demonstrated that microvascular dysfunction correlates with plasma insulin concentration, but not with plasma glucose, β cell function, age, body mass index, serum lipid concentration, or blood pressure (6). This correlation suggests that supraphysiological levels of insulin in prediabetes could contribute to the vascular dysfunction present in prediabetes, although the mechanism by which this occurs has not been previously defined.

One potential target for the adverse effect of insulin on the circulation is the erythrocyte. Erythrocytes deliver oxygen (O_2) throughout the body to meet the metabolic needs of tissues. The precise matching of O_2 supply with demand requires that blood flow be redistributed to areas of increased need. The erythrocyte can participate in this redistribution of blood flow and, thereby, O_2 supply by the release of adenosine triphosphate (ATP) when these cells are exposed to reduced O_2 tension (7–12). The ATP released from erythrocytes can stimulate the production of endothelium-derived vasodilators resulting in local increases in vascular caliber and, consequently, erythrocyte (O_2) supply to areas of tissue under increased metabolic demand (8–10). Under this hypothesis, any impairment of ATP release from the erythrocyte would hinder the matching of O_2 supply with demand and could contribute to the development of microvascular dysfunction. Importantly, the microvascular dysfunction associated with type II diabetes and prediabetes is characterized by inadequate O_2 delivery to tissues (1,13).

A signal transduction pathway culminating in the release of ATP from erythrocytes has been defined (14). A critical control point in this pathway is the intracellular concentration of cAMP. An accumulation of cAMP within the erythrocyte is required for ATP to be released from this cell (15). Within any cell type, the intracellular concentration of cAMP is regulated by phosphodiesterases (PDEs). When activated, PDEs hydrolyze cyclic nucleotides, enabling these enzymes to regulate many intracellular signaling pathways that involve cAMP (16). Although PDE activity in erythrocytes has been reported (17–22), the ability of these enzymes to regulate erythrocyte cAMP levels and ATP release has not been extensively studied. However, we have recently identified PDE3B in rabbit and human erythrocytes and demonstrated that inhibitors of PDE3 potentiate erythrocyte cAMP levels and increase ATP release (23,24).

In adipocytes and hepatocytes, insulin stimulates the hydrolysis of cAMP by activating the PDE3 family (25–27). Insulin, when bound to its receptor, activates a signal transduction pathway resulting in the phosphorylation and activation of PDE3 (28). Within this pathway, phosphoinositide – 3 kinase (PI3K) can either directly phosphorylate and activate PDE3 or stimulate the activation of protein kinase B that in turn phosphorylates and activates PDE3 (25,26,29,30). Erythrocytes possess PDE3, insulin receptors and PI3K (24,31–34) suggesting that insulin could stimulate cAMP hydrolysis in erythrocytes by activating PDE3. Since ATP release from erythrocytes requires an accumulation of cAMP within the cell, increased cAMP hydrolysis stimulated by insulin would be anticipated to limit ATP release.

Here we investigated the hypothesis that a mechanism by which the hyperinsulinemia of prediabetes inhibits ATP release from erythrocytes. We propose that the pathophysiological levels of insulin circulating in prediabetes activate PDE3 in the erythrocyte resulting in increased cAMP hydrolysis and impaired ATP release. This impaired erythrocyte ATP release could impair O_2 supply to tissues.

Materials and Methods

Isolation of human erythrocytes

Human blood was obtained by venipuncture using a syringe containing heparin (500 units). Blood was collected from 10 females and 9 males with an average age of 38 years (range 25–63 years).

After collection, whole blood was centrifuged at $500 \times g$ at 4°C for 10 minutes and the plasma, buffy coat, and uppermost erythrocytes were removed by aspiration and discarded. The remaining erythrocytes were washed three times in wash 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, final pH 7.4. Wright stains of erythrocytes prepared in this fashion revealed less than 1 leukocyte per 50 high power fields (approximately 8–10 leukocytes per mm^3). Previous studies demonstrate that these erythrocyte preparations are also devoid of platelet contamination (24). Cells were prepared on the day of use.

Determination of cAMP accumulation in erythrocytes in response to incubation with pharmacological agents in the absence and presence of insulin

Washed erythrocytes diluted to a 50% hematocrit (1 ml) were pretreated with a PI3K inhibitor, LY294002 (10 μM , Biomol), or a PDE3 inhibitor, cilostazol (100 μM), for 30 minutes in the absence or presence of 1 nM insulin (Humalog U 100[®], Eli Lilly) or its vehicle, saline. Following this pretreatment protocol, mastoparan 7 (Mas 7, 30 μM , Biomol), a direct activator of the G protein Gi (35), was added to the cell suspension for 30 minutes. The reaction was stopped by the addition of 4 ml of ice – cold ethanol containing 1 mM HCl. Samples were then prepared for the determination of cAMP concentration.

Measurement of cAMP

After addition of ice-cold ethanol/HCl, the samples were centrifuged at $14,000 \times g$ for 10 minutes at 4°C . The supernatant was removed and stored overnight at -20°C to precipitate the remaining proteins. This supernatant was centrifuged at $3,700 \times g$ for 10 minutes at 4°C . The final supernatant was removed and dried under vacuum centrifugation. The dried sample was reconstituted in assay buffer. The concentration of cAMP was determined using an enzyme immunoassay (GE Healthcare). Cell counts were obtained from erythrocytes at a hematocrit of 50%. The amounts of cAMP measured were normalized to 10^{10} cells/ml.

Determination of ATP release from erythrocytes in response to incubation with pharmacological agents in the absence and presence of insulin

Washed erythrocytes diluted to a 20% hematocrit were incubated with 10 μM mastoparan 7 (Mas 7, Genscript), following a 30 minute pretreatment with LY294002 (10 μM) or cilostazol (100 μM) in the absence or presence of 1 nM insulin (Humalog U 100[®]) or its vehicle, saline. The ATP concentration was determined at 5, 10, and 15 minutes after the addition of Mas 7. These time periods were chosen based on preliminary studies. The maximal response to Mas 7 in the absence or presence of insulin is reported.

Measurement of ATP

ATP was measured by the luciferin–luciferase technique (36). A 200 μL sample of 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; Sigma). The light emitted was detected using a luminometer (Turner Designs). A standard curve 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/mL.

Measurement of Total Intracellular ATP of Erythrocytes

A known number of erythrocytes were lysed in distilled water and diluted 8,000 fold in wash buffer (see above). ATP was measured as described above and the 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 $500 \times g$ at $4^\circ C$ for 10 minutes and the presence of free hemoglobin in the supernatant was determined by light absorption at a wavelength of 405 nm. If any free hemoglobin was detected, the data were not included to ensure that hemolysis was not influencing the ATP measurements.

Statistical Analysis

Statistical significance between 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 were reported as means $\pm$ the standard error of the mean (SEM).

Results

Effect of insulin on Mas 7-induced increases in cAMP and ATP release from human erythrocytes

Consistent with previous studies, incubation of erythrocytes with insulin (1 nM) had no effect on basal ATP release, but it attenuated Mas 7 – induced ATP release from these cells (Fig.1) (37). To ensure that decreases in ATP release could not be attributed to insulin – induced decreases in erythrocyte ATP content, total intraerythrocyte ATP was measured. Total ATP levels were not different in the absence and presence of 1 nM insulin (1.64 ± 0.17 and 1.52 ± 0.17 mM for control and insulin treated, respectively). Previously we reported that increases in cAMP are required for ATP release from human erythrocytes (15). To begin to investigate the mechanism by which insulin inhibits ATP release, we determined the effect of insulin on Mas 7 – induced increases in cAMP. Insulin (1 nM) had no effect on basal cAMP levels (data not shown). However, as depicted in Fig. 2, insulin did attenuate Mas 7 – induced increases in cAMP. These results support the hypothesis that insulin – induced inhibition of ATP release from human erythrocytes is associated with increased cAMP hydrolysis.

Effect of a PDE3 inhibitor on insulin-induced attenuation of cAMP accumulation within erythrocytes

Insulin has been shown to antagonize the effects of cAMP by stimulating the hydrolysis of this cyclic nucleotide (25–27). Insulin stimulates cAMP hydrolysis by activating a signal transduction pathway resulting in the phosphorylation and activation of PDE3, a PDE that has been shown to be present human and rabbit erythrocytes (24). To determine if inhibition of PDE3 can rescue insulin – induced inhibition of increases in cAMP stimulated by Mas 7, erythrocytes were pretreated with the selective PDE3 inhibitor cilostazol (100 μM) in the absence and presence of 1 nM insulin (38). As shown in Table 1, 100 μM cilostazol potentiated Mas 7 – induced cAMP accumulation. More importantly, in the presence of cilostazol, insulin did not inhibit Mas 7 – induced cAMP accumulation (Fig. 2).

Effect of a PI3K inhibitor on insulin – induced attenuation of cAMP accumulation within erythrocytes

Many of the cellular effects of insulin are mediated through its activation of phosphoinositide 3 – kinase (PI3K) (39). Indeed, activation of PDE3 by insulin and subsequent cAMP hydrolysis has been shown to require the activity of PI3K (29,30). To determine if insulin attenuates Mas 7 – induced increases in cAMP via a mechanism that requires activation of PI3K, erythrocytes were incubated with Mas 7 or Mas 7 plus insulin (1 nM) in the absence and presence of the selective PI3K inhibitor LY294002 (10 μ M). As shown in Table 1, 10 μ M LY294002 potentiated Mas 7 – induced cAMP accumulation in the absence of insulin. More importantly, in the presence of LY294002, insulin had no effect on Mas 7 – induced cAMP accumulation (Fig. 2).

Effect of a PDE3 or PI3K inhibitor on insulin – induced attenuation of ATP release from erythrocytes

To determine if inhibitors of PDE3 or PI3K prevent the effects of insulin on Mas 7 – induced ATP release from erythrocytes, cells were incubated with insulin in the absence and presence of either cilostazol (100 μ M) or LY294002 (10 μ M). ATP release was then stimulated by Mas 7. Cilostazol and LY294002 alone had no effect on Mas 7 – induced ATP release in the absence of insulin (data not shown). However, both cilostazol (Fig. 3) and LY294002 (Fig. 4) prevented the insulin – induced inhibition of ATP release produced by Mas 7. These results provide strong support for the hypothesis that insulin stimulates the hydrolysis of cAMP within the erythrocyte via PI3K-mediated PDE3 activation resulting, ultimately, in inhibition of ATP release from these cells.

Discussion

Adequate O₂ supply to tissue is required for the maintenance of energy production and normal cellular function (40). As the O₂ utilization of a tissue increases, this increase in demand must be met by an appropriate increase in blood flow and O₂ supply. The observation that, in skeletal muscle, blood flow increases selectively to regions of tissue with increased demand for O₂ implies that a mechanism exists to match O₂ supply with demand in that tissue (40–42).

We, and others, have suggested that erythrocytes have a critical role in the matching of O₂ supply with demand. The erythrocyte has long been known to participate in the delivery of O₂ by virtue of the hemoglobin it contains. More recently, the erythrocyte has been proposed to also regulate vascular caliber due to its ability to release the vasoactive nucleotide, adenosine triphosphate (ATP) (9–11,43,44). Erythrocytes release ATP in response to reduced O₂ tension (10,12), a stimulus this cell would encounter when traversing a tissue where O₂ utilization exceeds supply. The ATP released can then bind to purinergic receptors on vessel endothelium resulting in the production of endothelium – derived vasodilators and culminating in an increase in vascular caliber. Erythrocyte – derived ATP released in response to reduced O₂ tension would provide a mechanism for O₂ supply to meet precisely the O₂ demand of tissue. Under this hypothesis, failure of erythrocytes to release ATP in response to low O₂ tension would inhibit the ability of this cell to participate in the regulation of blood flow distribution to optimize O₂ delivery. Such a defect in erythrocyte function could contribute to the pathophysiology of vascular disease.

A signal transduction pathway that results in ATP release from erythrocytes exposed to reduced O₂ tension has been proposed. This pathway includes the heterotrimeric G protein G_i, adenylyl cyclase, cAMP, protein kinase A (PKA), and CFTR (14,15,45–47). Understanding the regulation of each component of this pathway would aid in determining the factors that

modulate not only ATP release from this cell, but also the ability of the erythrocyte to increase blood flow and O₂ delivery to meet tissue O₂ need.

ATP release in response to decreased O₂ tension requires the activation of Gi (12). Gi is classically considered an inhibitory G protein due to inhibition of adenylyl cyclase (AC) activity. However, it was shown that the $\beta\gamma$ subunits of Gi stimulate some isoforms of AC, including type II, an isoform that has been identified in rabbit and human erythrocytes (14,48–50). Importantly, direct activation of Gi by Mas 7 stimulates both cAMP production within the erythrocyte and ATP release from the cell (14,45,48), providing a pharmacological model of low O₂ tension – induced ATP release.

A critical control point in the pathway stimulating erythrocyte ATP release is the intraerythrocyte concentration of cAMP. An increase in cAMP within erythrocytes is required for ATP release (15). Phosphodiesterases (PDEs), via their ability to hydrolyze cAMP, not only regulate the intracellular concentrations of this second messenger, but also ultimately regulate biological processes that involve this cyclic nucleotide. Although PDE activity has been described in erythrocytes, it has not been well characterized. Recently, we identified PDE 3 in rabbit and human erythrocytes and have also demonstrated that inhibitors of this PDE family potentiate agonist – induced cAMP increases within these cells as well as ATP release (23,24).

Previously, we demonstrated that 1 nM insulin, a concentration of insulin that reflects postprandial levels in humans with prediabetes (51–54), inhibits ATP release from erythrocytes stimulated by reduced O₂ tension or Mas 7 (37). However, insulin had no effect on ATP release stimulated by the non – hydrolysable cAMP analog SpcAMPS, a direct activator of PKA (37). This suggests that insulin did not inhibit the activity of a component of the signal transduction pathway for ATP release distal to activation of PKA. We hypothesized that insulin inhibits ATP release from erythrocytes by stimulating the hydrolysis of cAMP within this cell. Here, we confirm that insulin inhibits Mas 7 – induced ATP release (Fig. 1). Additionally, we demonstrate that insulin inhibits Mas 7 – induced increases in cAMP within these cells (Fig. 2).

In other cell types, insulin stimulates cAMP hydrolysis via a signaling pathway that results in the phosphorylation and activation of PDE3 (25–27). To determine if PDE3 activity is required for insulin - induced inhibition of cAMP increases in erythrocytes, cells were preincubated with 1nM insulin and erythrocytes were treated with Mas 7 to stimulate cAMP increases in the presence or absence of the selective PDE3 inhibitor cilostazol. Figure 2 demonstrates that cilostazol prevented insulin – induced inhibition of increases of cAMP produced by Mas 7. This suggests that PDE3 regulates cAMP accumulation stimulated by activation of Gi and that PDE3 activity is required for insulin to inhibit cAMP increases in erythrocytes.

Insulin – induced activation of PDE3 has been reported to require the activity of phosphoinositide 3 – kinase (PI3K) (28–30). PI3K is present in erythrocytes (31), but this protein has not been related to PDE activity within this cell. To determine if PI3K is involved in the insulin – induced inhibition of cAMP accumulation in erythrocytes, cells were preincubated with insulin and cAMP increases were stimulated by Mas 7 in the presence and absence of the PI3K inhibitor LY294002. LY294002 prevented insulin – induced increases in cAMP produced by Mas 7 (Fig. 2). This demonstrates that PI3K is involved in the activation of PDE 3 by insulin in human erythrocytes. Taken together, these results support the hypothesis that insulin stimulates a signal transduction pathway within the erythrocyte involving PI3K that resulting in activation of PDE3 and, ultimately, increase cAMP hydrolysis.

Since insulin also inhibits Mas 7 – induced ATP release from erythrocytes (Fig. 1) (37), we next determined if inhibition of PDE3 or PI3K rescues Mas 7 – induced ATP release. We found

that inhibitors of either PDE3 or PI3K prevent the effect of insulin – induced inhibition of ATP release from these cells (Figs. 3, 4). These data provide support for the hypothesis that insulin – induced activation of erythrocyte PDE3 stimulates the hydrolysis of intracellular cAMP, inhibiting ATP release, and thereby hindering the ability of erythrocytes to stimulate vasodilation in response to reduced O₂ tension.

Inhibition of ATP release from erythrocytes would compromise the ability of this cell to stimulate vasodilation of blood vessels in response to reduced O₂ tension, ultimately impairing the participation of this cell in the matching of O₂ supply to O₂ need. Indeed, we demonstrated that treatment of erythrocytes with insulin inhibits the ability of this cell to stimulate vasodilation of blood vessels in response to reduced O₂ tension (37). Therefore, insulin – induced inhibition of ATP release from erythrocytes could impair the matching of O₂ supply to O₂ need.

Inadequate matching of O₂ supply and demand has been suggested to contribute to the development vascular complications in various diseases including, but not limited to, type II diabetes and prediabetes (1–3,13,55–57). Importantly, the severity of vascular complications in individuals with prediabetes correlates with plasma insulin levels (6), suggesting that high plasma insulin levels have a role in the development of the associated vascular complications. Under our hypothesis, the hyperinsulinemia present in prediabetes would result in increased binding of insulin to the erythrocyte, activation of PDE3, stimulation of cAMP hydrolysis, and ultimately, inhibition of ATP release when the cell is exposed to reduced O₂ tension (Fig. 5). Currently, the selective PDE3 inhibitor cilostazol is used successfully in the treatment of intermittent claudication (38), pain that develops in response to impaired blood flow and O₂ supply to muscle. Since cilostazol prevents the insulin – induced inhibition of erythrocyte ATP release, this suggests a heretofore unrecognized mechanism by which this drug could act to improve blood flow to skeletal muscle.

In summary, this study demonstrates that, in human erythrocytes : 1) insulin attenuates Mas 7 – induced cAMP accumulation and ATP release, 2) inhibition of PI3K or PDE3 prevents these effects of insulin. Mas 7 is a direct activator of Gi, the G protein required for ATP release from erythrocytes exposed to reduced O₂ tension (12). These results support the hypothesis that, by activating PDE3 in the erythrocyte, insulin inhibits erythrocyte cAMP accumulation and ATP release and, ultimately, could compromise the ability of this cell to stimulate vasodilation of blood vessels in response to reduced O₂ tension and thereby contribute to the peripheral vascular disease of prediabetes.

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Figure 1.

Effect of insulin on mastoparan 7 – induced ATP release from erythrocytes. Washed erythrocytes were incubated with mastoparan 7 (Mas 7, 10 μ M) 20 minutes after the addition of either insulin (1 nM) or its vehicle (saline) (n=9). The maximal ATP release in response to Mas 7 treatment was reported. Values are the means $\pm$ SE. * = greater than all other values ($P < 0.05$).

Figure 2.

Effect of cilostazol or LY294002 on mastoparan 7 – induced cAMP increases in erythrocytes in the absence and presence of insulin. Washed erythrocytes were incubated with cilostazol (Cilo, 100 μ M) or LY294002 (LY, 10 μ M) for 30 min in the absence or presence of insulin (1 nM) or its vehicle (saline) and then cAMP increases were stimulated by mastoparan 7 (Mas 7, 30 μ M). (n=9). cAMP levels were determined 30 minutes after the addition of Mas 7. Values are the means $\pm$ SE. * = greater than all other values ($P < 0.05$).

Figure 3.

Effect of cilostazol on mastoparan 7 – induced ATP release from human erythrocytes in the absence and presence of insulin. Washed erythrocytes were incubated with cilostazol (Cilo, 100 μ M) for 30 min in the absence or presence of insulin (1 nM) or its vehicle (saline) and then ATP release was induced by mastoparan 7 (Mas 7, 10 μ M) (n=8). The maximal ATP release in response to Mas 7 treatment was reported. Values are the means $\pm$ SE. * = greater than all other values ($P < 0.05$), NS = no significant difference between values.

Figure 4.

Effect of LY294002 on mastoparan 7 – induced ATP release from human erythrocytes in the absence and presence of insulin. Washed erythrocytes were incubated with LY294002 (LY, 10 μM) for 30 min in the absence or presence of insulin (1 nM) or its vehicle (saline) and then ATP release was induced by mastoparan 7 (Mas 7, 10 μM) (n=8). The maximal ATP release in response to Mas 7 treatment was reported. Values are the means ± SE. * = greater than all other values ($P < 0.05$), NS = no significant difference between values.

Figure 5.

Proposed mechanism by which insulin activates PDE3 in erythrocytes, inhibiting ATP release from these cells. G_{α_i} , heterotrimeric G protein G_i ; Mas 7, mastoparan 7; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CFTR, cystic fibrosis transmembrane conductance regulator; PI3K, phosphoinositide – 3 kinase; PDE3B, phosphodiesterase 3B; AMP, adenosine monophosphate

Table 1

Effect of cilostazol (100 μM) or LY294002 (10 μM) on mastoparan 7 (30 μM) – induced cAMP increase in human erythrocytes

cAMP (pmole/ 5×10^8 erythrocytes/ mm^3)		
Mas 7	Mas 7 + Cilostazol	Mas 7 + LY294002
2.06 ± 0.12 (n=9)	$2.36 \pm 0.27^*$ (n=7)	$2.23 \pm 0.24^*$ (n=7)

Mas 7, mastoparan 7; Values are mean $\pm$ SEM.

* significantly different from Mas 7 alone, ($P < 0.05$)