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Rabbit erythrocytes possess adenylyl cyclase type II that is activated by the heterotrimeric G proteins Gs and Gi

Randy Sprague, Elizabeth Bowles, Madelyn Stumpf, Gregory Ricketts, Alberto Freidman, Wei-Hsien Hou, Alan Stephenson, Andrew Lonigro

Department of Pharmacological and Physiological Science, Saint Louis University, School of Medicine, 1402 South Grand Blvd., Saint Louis, Missouri, USA 63104

Correspondence: Randy S. Sprague, e-mail: spraguer@slu.edu

Abstract:
Adenosine triphosphate (ATP) release from rabbit erythrocytes occurs in response to deformation or reduced oxygen tension. A signal transduction pathway that relates these stimuli to ATP release has been proposed. This pathway includes the heterotrimeric G proteins, Gs and Gi, adenylyl cyclase, protein kinase A, and the cystic fibrosis transmembrane conductance regulator. Importantly, adenylyl cyclase types II, IV and VII have been reported to be activated by both Gs and Gi. Here, we demonstrate that rabbit erythrocytes possess an adenylyl cyclase subtype that is activated both by the α subunit and the $\beta\gamma$ subunit of Gs and Gi, respectively. Washed rabbit erythrocytes released ATP when exposed to the β adrenergic receptor-mediated activator of Gs, isoproterenol (ISO, 10 μM, $n = 8$, $p < 0.05$) as well as in response to incubation with a direct activator of Gi, mastoparan 7 (MAS7, 10 μM, $n = 12$, $p < 0.05$). In contrast, an inactive mastoparan derivative, mastoparan 17 (MAS 17, 10 μM, $n = 6$) did not stimulate ATP release. Importantly, incubation of washed rabbit erythrocytes with either isoprotenerol (ISO) (10 μM, $n = 7$) or MAS7 (10 μM, $n = 11$) resulted in increases in cyclic adenosine monophosphate (cAMP) ($p < 0.01$). Western analysis was used to determine if an adenylyl cyclase capable of being activated by both Gs and Gi was a component of rabbit erythrocyte membranes. We identified adenylyl cyclase type II with two antibodies generated against different epitopes of the protein. These results provide support for the hypothesis that, in rabbit erythrocytes, activation of either Gs or Gi results in the stimulation of adenylyl cyclase resulting in increases in cAMP leading, ultimately, to the release of ATP.

Key words:
red blood cell, adenosine triphosphate, adenylyl cyclase, mastroparan 7, isoproterenol

Introduction

Previously, we reported that rabbit and human erythrocytes stimulate endogenous nitric oxide (NO) synthesis in the circulation *via* the release of adenosine triphosphate (ATP) [27, 28, 30]. ATP released from circulating erythrocytes can bind to purinergic receptors found on the endothelium that are linked to signal transduction pathways that lead, ultimately, to the synthesis and release of NO [6, 13]. Indeed, it has been shown that rabbit and human erythrocytes release ATP in response to the physiological stimuli of reduced oxygen tension [5, 10, 18, 20, 28] and mechanical deformation [26, 28, 30].

The mechanism by which ATP is released from erythrocytes has also been investigated. It has been reported that erythrocytes of rabbits and humans possess a signal transduction pathway that relates ATP release to deformation and exposure to reduced oxygen

tension. This pathway has been reported to include the heterotrimeric G proteins Gs [24] and Gi [22, 23], adenylyl cyclase [29], protein kinase A [29] and the cystic fibrosis transmembrane conductance regulator (CFTR) [16, 26]. Importantly, under the proposed pathway, activation of either Gs or Gi was associated with the release of ATP from erythrocytes [22–24]. Heterotrimeric G proteins are membrane associated guanosine triphosphate (GTP)-binding proteins that contain α, β, and γ subunits [10, 20]. When activated, the α subunit dissociates from the βγ complex. The α subunit and the βγ complex, can then regulate, either individually or synergistically, the catalytic activity of available adenylyl cyclase (AC) isoforms present in the cell [1, 9, 10, 18, 20, 31]. The α subunit of Gs activates all known isoforms of AC [10, 20]. In the case of other heterotrimeric G proteins, in addition to the activity of the α subunit, it is now recognized that the βγ subunit may be capable of activating at least three of the eight membrane associated isoforms of AC (subtypes II, IV and VII, Tab. 1) [1, 9, 18, 31]. The heterotrimeric G proteins most clearly associated with this property are of the Gi/o subclass [1, 9, 31]. The heterotrimeric G protein, Go, is not found in rabbit erythrocyte [22], however, Giα subtypes 1, 2 and 3, are present [22]. Thus, rabbit erythrocytes possess a heterotrimeric G protein that is capable of stimulating some subtypes of adenylyl cyclase *via* the action of its βγ subunit.

Here we investigated the hypothesis that rabbit erythrocytes possess at least one adenylyl cyclase, specifically type II, that can be stimulated by activation of Gs as well as Gi. Moreover, we wished to demonstrate that pharmacological activation of either of these G proteins results in both ATP release from and 3'-5'-cyclic adenosine monophosphate (cAMP) accumulation in rabbit erythrocytes.

Materials and Methods

Preparation of erythrocytes

Animals (male, New Zealand white rabbits, 2 to 3 kg) were anesthetized (ketamine, 0.25 ml/kg and xylazine, 1.5 mg/kg, *im,* followed by pentobarbital 15 mg/kg *iv*) *via* a catheter placed in an ear vein. Following tracheostomy, animals were mechanically ventilated (tidal volume 10 ml/kg, rate 20–25 breaths/min, Harvard ventilator). A catheter was placed into a carotid artery, heparin (500 units *iv*) was administered and, after 10 min, animals were exsanguinated. Immediately after collection of blood, erythrocytes were separated from other formed elements and plasma by centrifugation at 500 × g at 4°C for 10 min. The supernatant and buffy coat were removed by aspiration. The packed erythrocytes were resuspended and washed 3 times in a physiological salt solution (PSS) [in mM; 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl and 21.0 tris(hydroxymethyl)-aminomethane (Tris) and 5.5 dextrose with 0.5% bovine serum albumin, pH 7.4]. Erythrocytes were prepared on the day of use. The protocol for removal of blood from rabbits was approved by the Animal Care Committee of Saint Louis University.

Preparation of erythrocyte membranes

Washed erythrocytes (2 ml) were added to 200 ml of a hypotonic buffer solution (in mM; 2.0 ethylenediamine-tetraacetic acid (EDTA) and 5 tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) with pH adjusted to 7.4) and stirred vigorously for 20 min at 4°C. The mixture was centrifuged at 23,700 × g for 10 min. The supernatant was discarded and the membranes were washed twice in the hypotonic buffer. After the second wash, the membrane fraction was passed through a 23 gauge needle three times, aliquotted and stored at –80°C. Protein concentration was determined with the bicinchoninic acid (BCA) protein assay (Pierce).

Identification of adenylyl cyclase type II in erythrocyte membranes

Membranes were solubilized in SDS sample buffer [8% sodium dodecyl sulfate (SDS), 60% glycerol, 0.25 M Tris HCl (pH 6.8), 0.004% bromophenol blue, and 400 mM dithiothreitol] and boiled for 5 min before loading onto a 5% pre-cast Tris-HCl Ready Gel (Bio-Rad). After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked overnight with 5% non-fat dry milk in phosphate-buffered saline containing 0.1% Tween-20 and then incubated with rabbit polyclonal antibodies to adenylyl cyclase II (Santa Cruz). Membranes were then incubated with donkey anti-rabbit IgG-horseradish peroxidase (Amersham) and exposed to enhanced chemiluminescence (ECL, Amersham).

Measurement of ATP and hemoglobin

ATP was measured by the luciferin-luciferase technique [2, 27, 28] which utilizes the ATP concentration-dependence of light generated by the reaction of ATP with firefly tail extract. Sensitivity was augmented by addition of synthetic D-luciferin to the crude firefly tail extract. A 200 μl sample of the erythrocyte suspension was injected into a cuvette containing 100 μl crude firefly tail 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). An ATP standard curve was obtained on the day of each experiment. To exclude the presence of significant hemolysis, free hemoglobin was determined after ATP measurements. Samples were centrifuged at 500 × g at 4°C for 10 min and the presence of hemoglobin in the supernatant was determined by light absorption at a wavelength of 405 nm [33]. In response to stimulation with pharmacological agonists, the ATP signal increased in the absence of any increase in light absorption in the range consistent with the detection of hemoglobin. All data from experiments in which free hemoglobin increased were excluded. To ensure that the results of the ATP assay were not altered by the agents with which erythrocytes were incubated, the effects of mastoparan 7 (MAS7) and isoproterenol (ISO) on ATP measurement were determined. These agents, at the concentrations used in this study, did not alter the sensitivity of the assay for authentic ATP (data not shown). Amounts of ATP released were normalized to the number of erythrocytes present in suspension at a hematocrit of 20% (4×10^5 cells/mm^3).

Measurement of cAMP

For determination of cAMP, 1 ml of erythrocyte suspension was added to 4 ml of ice cold ethanol containing 1 mM HCl 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 then centrifuged a second time at 3,700 × g for 10 min at 4°C. The supernatant was removed and dried under vacuum centrifugation. Concentrations of cAMP were then determined with the cAMP Enzyme Immunoassay Biotrak (EIA) System (Amersham Biosciences). Amounts of cAMP measured were normalized to the number of erythrocytes present in suspension at a hematocrit of 50% (1×10^7 cells/mm^3).

Incubation of erythrocytes with agents that activate Gs or Gi

The effect of Gs activation on cAMP accumulation and ATP release from erythrocytes was investigated by incubation of cells suspended in PSS (hematocrit 20%) with isoproterenol (ISO, 10 μM, n = 8). ATP measurements were made in the absence of the agonist as well as 5, 10 and 15 min after addition of ISO. In the case of cAMP, measurements were made 10 min after the addition of ISO (10 μM, n = 7) to erythrocytes suspended in PSS at a hematocrit of 50%. In all cAMP studies, 3-isobutyl-1-methyl xanthine (IBMX, 100 μM, dissolved in ethanol and diluted with phosphate buffered saline, pH 7.4) was included to prevent cAMP degradation.

The effect of activation of Gi on ATP release in erythrocytes was investigated by incubation of cells suspended in PSS (hematocrit 20%) with mastoparan 7 (MAS7, 10 μM, n = 12) [12, 15, 22]. ATP measurements were made in the absence of the agonist as well as 5, 10 and 15 min after addition of MAS7. Measurements cAMP were made 30 min after the addition of MAS7 (10 μM, n = 11) to erythrocytes suspended in PSS at a hematocrit of 50%. To establish that MAS7-induced ATP release was related to the activation of Gi, in 12 additional studies, erythrocytes were treated with the inhibitor of Gi activation, pertussis toxin (PTX, 100 ng/ml), for 2 h before determination of MAS7-induced ATP release. Finally, the effect of an inactive mastoparan derivative, mastoparan 17 (MAS17, 10 μM, n = 6) on ATP release from erythrocytes was determined.

Statistics

Statistical significance between experimental periods was determined with an analysis of variance. In the event that the F ratio indicated that changes had occurred, a least significant difference test was used to identify individual differences. p values of 0.05 or less were considered statistically significant. Results are reported as means ± SEM.

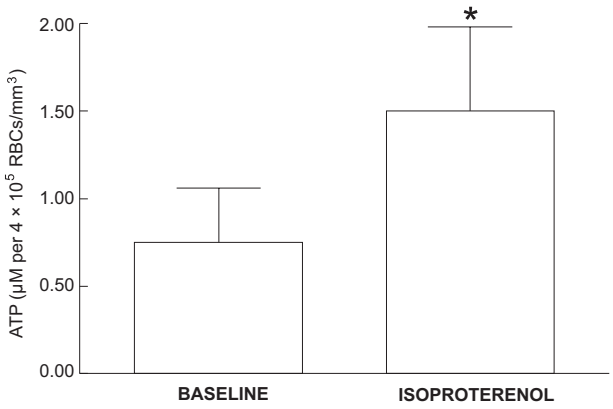

Fig. 1. Effect of isoproterenol on ATP release from rabbit erythrocytes. Washed erythrocytes (20% hematocrit) were incubated with isoproterenol (10 μM, n = 8). ATP release was determined before (BASELINE) and 5, 10 and 15 min after addition of isoproterenol. The value for ISOPROTERENOL is the peak value obtained. * $p < 0.05$, different from baseline

Results

Effect of activation of Gs with ISO on ATP release from and cAMP accumulation in erythrocytes

The effect of incubation of erythrocytes with ISO on ATP release is depicted in Figure 1. The maximum increase in ATP concentration occurred 10 ± 1 min after administration of ISO. In response to ISO, erythrocyte cAMP increased by 36 ± 7% (Tab. 1, $p < 0.01$).

Tab. 1. Concentration of cAMP in erythrocytes (pmole per 1 x 10^7 erythrocytes/mm^3)

GROUP	BASELINE	STIMULATED
isoproterenol (ISO, n = 7)	3.11 ± 0.36	4.19 ± 0.54*
mastoparan 7 (MAS7, n = 11)	1.12 ± 0.06	1.34 ± 0.06*

Values are mean ± SEM. * – different from value for respective BASELINE, $p < 0.01$.

Effect of activation of Gi with MAS7 on ATP release from and cAMP accumulation in erythrocytes

The effect of incubation of erythrocytes with MAS7 on ATP release is depicted in Figure 2A. The maximal increase in ATP concentration occurred 8 ± 1 min after administration of MAS7. The results of two additional studies provide support for the hypothesis that MAS7 acted *via* stimulation of Gi. First, MAS7-induced increases in ATP release were prevented by pre-incubation of erythrocytes with pertussis toxin, an inhibitor of the activation of Gi (Fig. 2A). Second, incubation of erythrocytes with the inactive mastoparan derivative, MAS17 was not associated with any increase in ATP release, i.e. ATP release was not a non-selective response to exposure of erythrocytes to protein (Fig. 2B). In response to incubation with MAS7, cAMP concentration in erythrocytes increased by 22 ± 8 % (Tab. 1, $p < 0.01$).

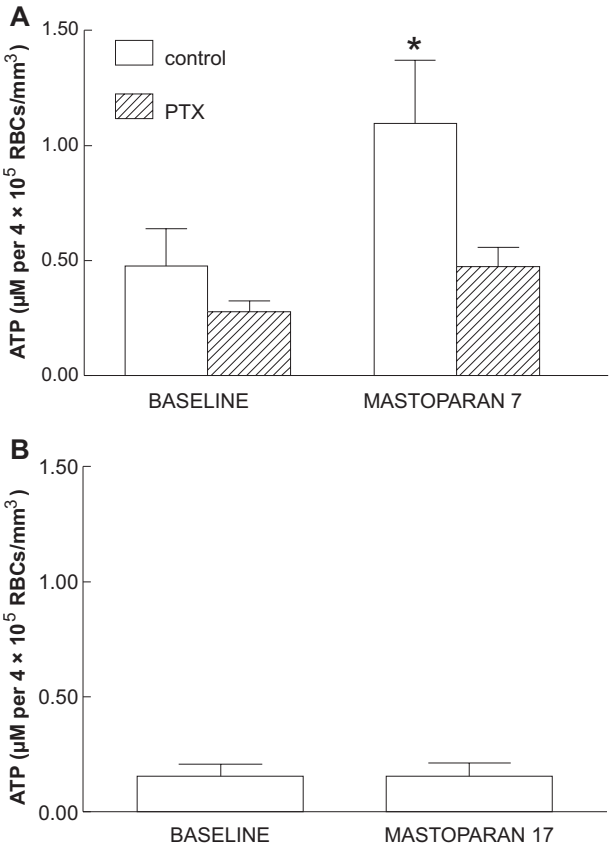

Fig. 2. Effect of mastoparan 7 and mastoparan 17 on ATP release from rabbit erythrocytes. (**A**) Washed erythrocytes (20% hematocrit) were incubated with mastoparan 7 (10 μM, n = 12) in the absence (open bars) and presence (cross hatched bars) of pertussis toxin (PTX, 100 ng/ml for 2 h). ATP release was determined before (BASELINE) and 5, 10 and 15 min after addition of mastoparan 7. The values for mastoparan 7 are the peak values obtained. (**B**) Washed erythrocytes (20% hematocrit) were incubated with mastoparan 17 (10 μM, n = 6). ATP release was determined before (BASELINE) and 5, 10 and 15 min after addition of mastoparan 17. The value for MASTOPARAN 17 is the peak value obtained. * $p < 0.05$, different from BASELINE and MASTOPARAN 7 after PTX

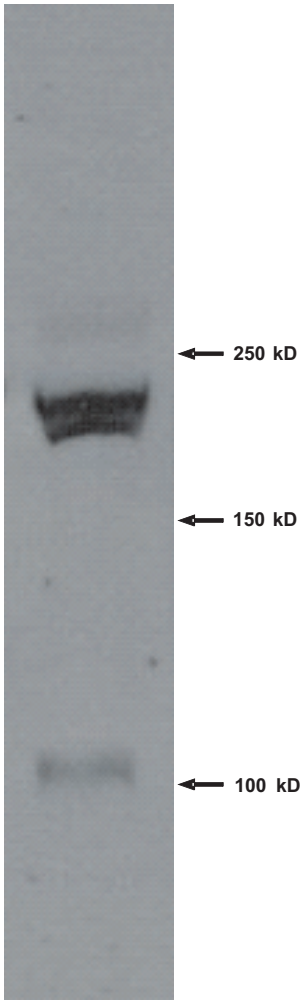

Fig. 3. Identification of adenylyl cyclase type II in rabbit erythrocyte membranes. Membranes were prepared as described and the protein was resolved using 5% pre-cast Tris-HCL Ready Gels. Protein was transferred to a PVDF membrane and processed with rabbit polyclonal anti-adenylyl cyclase type II antibody. The gel depicted is representative of 5 individual studies in which 25 µg of membrane protein were loaded

Identification of adenylyl cyclase (AC) type II as a component of rabbit erythrocyte membranes

Erythrocyte membrane preparations from 5 rabbits were studied *via* Western blotting to determine the presence of AC type II. Two distinct bands were identified by an antibody generated against the c-terminal domain of this AC subtype, one at an apparent molecular weight of ~200 kD and a second band at ~110 kD. A typical gel is depicted in Figure 3. Importantly, we determined that a second rabbit polyclonal antibody directed against an internal epitope of AC type II identified the same ~200 kD band (data not shown).

Discussion

Erythrocyte-derived ATP has been shown to be a determinant of vascular resistance in the pulmonary circulation [27, 28, 30] and skeletal muscle [3, 8, 17] as well as in isolated resistance vessels [6]. The ability of this ATP to decrease vascular resistance has been reported to result from the stimulation of vascular NO synthesis [7, 27, 30]. Erythrocytes of humans, rats, rabbits and hamsters release ATP in response to reduced O_2 tension and deformation [2, 7, 28, 30]. Moreover, a signal-transduction pathway that relates these stimuli to ATP release has been proposed and includes the cystic fibrosis transmembrane conductance regulator (CFTR) [17, 26], protein kinase A (PKA) [29], AC and cAMP [29] as well as the heterotrimeric G proteins Gs [24] and Gi [22, 23]. Importantly, within this pathway, *activation* of Gi *stimulates* ATP release (Fig. 4) [22, 23].

When heterotrimeric G proteins are activated, they dissociate into α and βγ subunits [10, 20]. The α subunit of the G protein, Gs, can then activate all AC isoforms [10, 20]. In contrast, the α subunit of the G protein, Gi, can interact with some AC isoforms resulting in their inactivation [1, 9, 18, 31]. Hence, traditionally, Gs is referred to as a "stimulatory" G protein while Gi is considered to be an "inhibitory" G protein. Recently, the concept that Gi is solely an inactivator of AC has been called into question by reports that, in addition to the inhibitory effect of α subunit of Gi on some isoforms of AC, the βγ subunit of Gi is capable of activating other isoforms, specifically those of the II, IV and VII subtypes [1, 9, 31].

The hypothesis that heterotrimeric G proteins of the Gi subtype are involved in deformation-induced release of ATP from erythrocytes is supported by reports that these G proteins are activated when endothelial cells as well as other cell types are exposed to a deforming force [11, 14, 21]. In addition, it was shown previously that pre-incubation of rabbit erythrocytes with pertussis toxin, which inhibits activation of Gi by preventing its dissociation into the constituent α and βγ subunits, also inhibits ATP release in response to both deformation and exposure to reduced oxygen tension [22, 23]. One interpretation of this finding is that these physiological stimuli resulted in activation of Gi, resulting in its dissociation into the component α and βγ subunits and subsequent stimulation of AC resulted in increased cAMP and, ulti-

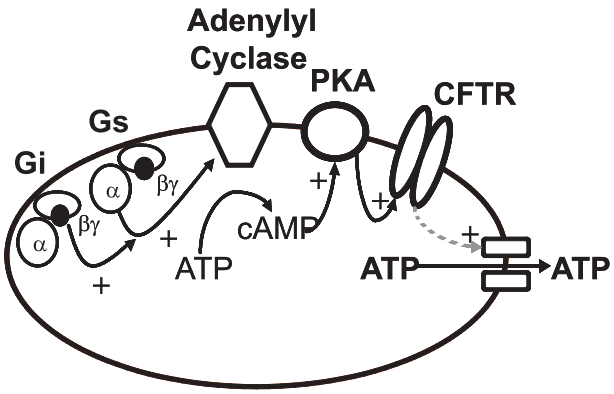

Fig. 4. A proposed signal transduction pathway for ATP release from rabbit erythrocytes. + – stimulates, Gs and Gi – heterotrimeric G proteins (α, β and γ subunits of Gs and Gi), PKA – protein kinase A, CFTR – cystic fibrosis transmembrane conductance regulator. The final ATP conduit is not defined

mately, ATP release. Here we report that incubation of rabbit erythrocytes with either isoproterenol, a selective β adrenergic receptor agonist that activates Gs (Fig. 1) or MAS7, a derivative of mastoparan which activates Gi (Fig. 2A), stimulate both ATP release (Figs. 1 and 2A) and cAMP accumulation (Tab. 1) in rabbit erythrocytes. The finding that pre-treatment of erythrocytes with PTX inhibited MAS7-induced ATP release (Fig. 2A) and that the inactive mastoparan derivative, MAS17, had no effect on ATP release (Fig. 2B) provide additional support for the hypothesis that the observed effects of MAS7 are mediated by activation of the heterotrimeric G protein, Gi. Moreover, these results suggest that, in these cells, activation of Gi results in stimulation of AC activity *via* the activity of the associated βγ subunit.

If activation of Gi results in the stimulation of AC activity, it is important to demonstrate that an AC subtype that is stimulated by the βγ subunit of this heterotrimeric G protein is a component of rabbit erythrocyte membranes. Here, using Western analysis, we report for the first time that rabbit erythrocytes possess at least one AC isoform that is activated by βγ subunits of Gi, namely, AC type II (Fig. 3). The antibody to AC type II reacted with two distinct proteins based on apparent molecular weight; one at ~200 kD and another at ~110 kD. The molecular weight of AC type II would be predicted to approximate 120 kD based upon analysis of amino acid composition. However, it has been reported that both fragments with lower apparent molecular weight as well as higher molecular weight complexes (200–250 kD) [19, 25, 32] may be found in various cell types. The higher molecular weight complexes have been suggested to result from dimerization, association with other proteins or glycosylation [19, 25, 32]. Importantly, it was reported that, in human myometrium, AC was found predominantly at an apparent molecular weight of ~200 kD [25]. In the work presented here we found that the antibody to AC type II recognized two bands. The denser band was determined to have an apparent molecular weight of ~200 kD, consistent with the possibility that the protein is either associated with other proteins in the signaling cascade, dimerized or glycosylated in erythrocytes membranes. The finding of a second band at an apparent molecular weight of ~110 kD is consistent with identification of the AC monomer [32].

In summary, in the work presented here we have demonstrated that activation of heterotrimeric G proteins of both the Gs and Gi subtypes results in ATP release from rabbit erythrocytes. Moreover, we have shown that the release of ATP is accompanied by increases in intracellular cAMP levels suggesting that activation of both Gs and Gi results in stimulation of AC activity. Finally, we have identified the presence of AC type II in rabbit erythrocyte membranes. The latter finding is important in that this is the first identification of any AC in an erythrocyte membrane and that the isoform identified, AC type II, is one that is activated by both the α subunit of Gs and the βγ subunit of Gi. These results provide new information regarding the nature of the signal transduction pathway that relates physiological stimuli to ATP release from erythrocytes. A better understanding of this pathway will permit the development of new hypotheses regarding the control of ATP release from erythrocytes in health and disease.

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