The effect of iloprost on the ADP-ribosylation of $G_s\alpha$ (the α -subunit of G_s)

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Treatment of platelets with a prostacyclin analogue, iloprost, decreased the cholera-toxin-induced ADP-ribosylation of membrane-bound $G_s\alpha$ (α -subunit of G-protein that stimulates adenylate cyclase; 42 kDa protein) and a cytosolic substrate (44 kDa protein) [Molina y Vedia, Reep & Lapetina (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5899–5902]. This decrease is apparently not correlated with a significant change in the quantity of membrane $G_s\alpha$, as detected by two $G_s\alpha$ -specific antisera. This finding contrasts with the suggestion in a previous report [Edwards, MacDermot & Wilkins (1987) *Br. J. Pharmacol.* **90**, 501–510], indicating that iloprost caused a loss of $G_s\alpha$ from the membrane. Our evidence points to a modification in the ability of the 42 kDa protein to be ADP-ribosylated by cholera toxin. This modification of $G_s\alpha$ might be related to its ADP-ribosylation by endogenous ADP-ribosyltransferase activity. Here we present evidence showing that $G_s\alpha$ was ADP-ribosylated in platelets that had been electropermeabilized and incubated with [α - 32 P]NAD $^{+}$. This endogenous ADP-ribosylation of $G_s\alpha$ is inhibited by nicotinamide and stimulated by iloprost.

INTRODUCTION

Prostacyclin and adenosine inhibit platelet activation, and this action is mediated by the stimulation of adenylate cyclase. Decreased platelet responsiveness to prostacyclin, after exposure of platelets to an agonist that interacts with the prostacyclin receptor, has previously been reported [1]. This homologous desensitization correlates with a decrease in the number of plasma-membrane receptors [2]. Heterologous desensitization has also been shown: prolonged incubation of platelets with iloprost, a prostacyclin analogue, results in a decrease in adenylate cyclase stimulated by adenosine A_2 -receptor agonists [3]. This heterologous desensitization can be explained by an apparent alteration in the transducer or $G_s\alpha$ protein that relates the receptor to activation of adenylate cyclase.

Desensitization by iloprost in platelet membranes, or by prostaglandin E in liver membranes, is associated with a decrease in ADP-ribosylation of $G_s\alpha$ by cholera toxin [3,4]. These results were interpreted as a loss of $G_s\alpha$ from the plasma membranes of platelets [3] or a qualitative change in the $G_s\alpha$ in liver [4]. In the present study, we attempted to determine the fate of $G_s\alpha$ in platelets treated with iloprost. Our information indicates that $G_s\alpha$ is not lost from the membrane, but changes its ability to be ADP-ribosylated by cholera toxin. This loss of ADP-ribosylation by cholera toxin seems to be caused by endogenous ADP-ribosylation of $G_s\alpha$.

MATERIALS AND METHODS

Materials

Cholera toxin (A-subunit) and pertussis toxin were obtained from List Biochemicals. Nicotinamide was from Sigma. Affinity-purified goat antibody to rabbit IgG [F(ab) $_2$ fragment] labelled with 125 I and [α - 32 P]NAD $^{+}$ (1000 Ci/mmol) were from NEN. $G_s\alpha$ -584 and $G_s\alpha$ -A-

572 antisera were kindly provided by Dr. Susanne Mumby and Dr. Alfred Gilman, Department of Pharmacology, Southwestern Medical Centre, Dallas, TX, U.S.A. Iloprost was a gift from Dr. Claus-Steffen Sturzebecher, Schering, Berlin, Germany.

Preparation of platelet membranes

Platelet membranes were prepared as previously described [3]. Briefly, platelets from 5 ml of platelet-rich plasma were disrupted in 5 ml of 5 mM-Tris/HCl buffer, pH 7.4, containing 0.25 mM-EDTA, by using a Dounce homogenizer. The membranes and supernatant were separated by centrifugation at 100 000 *g* for 40 min, and the membrane pellet was washed twice by resuspension in 5 ml of the same Tris/HCl/EDTA buffer. The washes were discarded. The pellet was finally resuspended in 200 μ l of 50 mM-Tris/HCl buffer, pH 7.4, containing 0.25 mM-EDTA.

[α - 32 P]ADP-ribosylation of proteins by cholera toxin

The cholera-toxin-catalysed reaction mixture [3] contained 15 mM-Tris/HCl buffer, pH 7.4, 1 mM-ATP, 15 mM-glycine, 2.5 mM-MgCl $_2$, 10 mM-thymidine, 6 μ M-NAD $^{+}$, 5 μ Ci of [α - 32 P]NAD $^{+}$ /assay, 100 μ M-GTP, 300 mM-potassium phosphate, pH 7, 10 μ g of platelet protein and 20 μ g of activated cholera-toxin A-subunit/assay. After incubation for 30 min at 37 °C, samples were precipitated with 1 ml of chilled 10% (w/v) trichloroacetic acid, and then centrifuged for 20 min at 2000 *g*. The pellets were finally washed with 1.5 ml of diethyl ether, and proteins were resolved by electrophoresis in SDS/10% polyacrylamide gels [5].

[α - 32 P]ADP-ribosylation of proteins by pertussis toxin

This was done as previously described [6].

Preparation of immunoblots

This was performed by procedures previously published by Harris *et al.* [7] and Mumby *et al.* [8].

Abbreviation used: $G_s\alpha$, α -subunit of GTP-binding protein that stimulates adenylate cyclase.

Briefly, 50 μg samples of platelet proteins per lane were resolved in SDS/10% polyacrylamide gels, and then they were electrophoretically transferred to nitrocellulose blots. Blots were treated with buffer containing 5% (w/v) non-fat dry milk, 0.2% Nonidet P40, 0.025% NaN_3 , 50 mM-Tris/HCl, pH 8, 2 mM- CaCl_2 and 80 mM-NaCl (Buffer A), followed by incubation for 1 h with specific antisera in Buffer A and three 20 min washes. Antibody binding was detected by incubation of the blots with affinity-purified goat antibody F(ab)₂ fragment to rabbit IgG labelled with ^{125}I (2×10^5 c.p.m./ml) in Buffer A. The blots were washed three times with Buffer A, and twice more in the same buffer without milk and Nonidet P40. Blot autoradiographs were obtained with Kodak XAR-5 film after overnight exposure.

Electropermeabilization of platelets

Platelets from 50 ml of fresh human blood were washed twice with buffer containing 113 mM-NaCl, 4.3 mM- K_2HPO_4 , 24.4 mM- NaH_2PO_4 , 4.3 mM- Na_2HPO_4 , 5 mM-glucose and 1 mM-EGTA, pH 7 (Buffer B), and resuspended in 1 ml of buffer [9] containing 150 mM-potassium glutamate, 20 mM-Pipes (potassium salt), 5 mM-sodium ATP, 7 mM-magnesium acetate, 5 mM-glucose and 1 mM-EGTA. The pH was adjusted to 6.6 by addition of KOH. The platelets were rendered permeable by ten exposures of this suspension to an intense electric field ($20 \text{ kV} \cdot \text{cm}^{-1}$). Platelets were divided into two fractions of 400 μl each, to which 500 μCi of $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ was added. In one of these fractions the concentration of nicotinamide was adjusted to 50 mM. After incubation for 1 h the cells were washed with $2 \times 20 \text{ ml}$ of Buffer B. Platelets were then resuspended in platelet-poor plasma from the same donor, containing 100 mM-Hepes, pH 7.2, and incubated for 24 h with or without 10 μM -iloprost and 50 mM-nicotinamide. Membranes were prepared after washing the cells twice with Buffer B and resuspending the platelets in hypo-osmotic buffer containing 5 mM-Tris/HCl, pH 7.4, and 0.25 mM-EDTA. After one cycle of freezing and thawing, the particulate was pelleted by centrifuging for 30 min at 100000 g, and washed twice with hypo-osmotic buffer.

Separation of $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ and $[\alpha\text{-}^{32}\text{P}]\text{ADP-ribose}$ by h.p.l.c.

This was done as previously reported [10]. Briefly, the first supernatant obtained in the procedure described above was precipitated with 10% trichloroacetic acid. The supernatant was extracted four times with water saturated with ether. The aqueous phase was adjusted to pH 7 with conc. NH_3 , and a sample containing 4×10^5 c.p.m. was injected on the column. $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ and $[\alpha\text{-}^{32}\text{P}]\text{ADP-ribose}$ were separated by h.p.l.c. on a Whatman Partisil 10 SAX column at a flow rate of 1 ml/min. The products were eluted with a concave gradient containing 0–1 M ammonium phosphate, pH 3.8, over 4000 s, followed by isocratic elution at 1 M-ammonium phosphate for 1000 s. Radioactivity was monitored with a Flow one Beta (model BD) flow detector (Radiomatic Instruments, Tampa, FL, U.S.A.), and the data were processed with a standard microcomputer.

The $[\alpha\text{-}^{32}\text{P}]\text{ADP-ribose}$ standard was produced by hydrolysis of $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ with NADase from *Neurospora crassa* (Sigma) using 0.08 unit of enzyme per assay in 100 μl of solution containing 100 mM-potassium phosphate buffer, pH 7.5, 50 μM -NAD⁺ and $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$

(0.1 μCi) for 30 min at 37 °C. Under this condition complete conversion of NAD⁺ into ADP-ribose occurred.

RESULTS

Effect of iloprost on cholera-toxin-induced ADP-ribosylation of membrane and cytosolic proteins

In a recent report [6] we showed that cholera toxin ADP-ribosylates a platelet cytosolic 44 kDa peptide that is not recognized by two $\text{G}_s\alpha$ -specific antisera. The platelet membrane has a 42 kDa peptide that is ADP-ribosylated by cholera toxin, and it is recognized by two $\text{G}_s\alpha$ antisera [6]. Fig. 1 shows the cholera-toxin-induced ADP-ribosylation of membranes from human platelets that were pretreated with iloprost for different times. Iloprost produced a decrease in cholera-toxin-induced ADP-ribosylation of $\text{G}_s\alpha$. This was not observed when platelets were pretreated with dibutyryl cyclic AMP (1 mM) instead of iloprost (results not shown). The decrease in the membrane-bound 42 kDa peptide was evident after treatment for 4 h with iloprost, and it was much greater after 24 h (Fig. 1). Previously, a similar observation was reported [3,6] on the decrease in ADP-ribosylation of the membrane-bound substrate of cholera toxin after 24 h of treatment with iloprost. The cholera-toxin-induced ADP-ribosylation of the cytosolic 44 kDa peptide was also decreased by pretreatment with iloprost [6].

Fig. 1. Effect of iloprost on the cholera-toxin-induced ADP-ribosylation of a membrane-bound substrate (42 kDa)

All assay incubations of platelet-rich plasma were for a total of 24 h. Treatment with iloprost was for the times indicated in the Figure, and treatment was made just before completion of the 24 h incubation period. Then platelets were separated, lysed and subjected to ADP-ribosylation as described in the Materials and methods section. The blot autoradiograph was obtained after overnight exposure.

Fig. 2. Detection of the 42 kDa protein by $G_s\alpha$ antisera 584
Detection of membrane-bound 42 kDa protein with $G_s\alpha$ antisera 584 after pretreatment of platelets with iloprost for various times was as detailed in Fig. 1.

Iloprost does not decrease the content of $G_s\alpha$ in platelet membranes

The dramatic decrease in cholera-toxin-induced ADP-ribosylation observed in the membrane-bound 42 kDa peptide was not correlated with a loss of the peptide from the membrane as determined with the $G_s\alpha$ antisera 584 (Fig. 2) and A-572 (results not shown). Pretreatment of platelets with iloprost neither decreased the amount of $G_s\alpha$ in the membrane (Fig. 2) nor translocated the protein to the cytosol [11]. A protein (23 kDa) immunologically related to $G_s\alpha$ is translocated (Fig. 2), as previously shown [11].

Effect of nicotinamide on ADP-ribosylation induced by cholera toxin and pertussis toxin

Endogenous ADP-ribosyltransferase activity, which is mostly cytosolic in human platelets, was previously described [6,10,12]. Nicotinamide is an inhibitor of endogenous ADP-ribosylation [13,14]. Therefore we preincubated platelets without and with 50 mM-nicotinamide to see if the pattern of ADP-ribosylation induced by cholera toxin or pertussis toxin was affected. Fig. 3 shows that preincubation of platelets for 24 h with nicotinamide increased the subsequent ADP-ribosylation of the membrane 42 kDa protein. Moreover, the effect of iloprost was slightly reversed by preincubation with nicotinamide (Fig. 3). On the other hand, the substrate of pertussis-toxin-induced ADP-ribosylation was not affected by nicotinamide or iloprost (Fig. 4).

Iloprost increases the ADP-ribosylation of $G_s\alpha$ in electroporated platelets

We electroporated platelets in a buffer previously described [9] that resembled the intracellular milieu. [32 P]NAD $^{+}$ was then added to the permeabilized platelets, and they were further incubated for 1 h in either the absence or the presence of 50 mM-nicotinamide. After they were washed, the platelets were incubated for 24 h

Fig. 3. Effect of pretreatment with iloprost and nicotinamide on the ADP-ribosylation of membranes by cholera toxin

Platelet-rich plasma was treated with 50 mM-nicotinamide or 50 mM-nicotinamide plus 10 μ M-iloprost for 24 h. Then platelets were separated and lysed, and the membranes were subjected to ADP-ribosylation in the presence of cholera toxin as described in the Materials and methods section. Gel autoradiograph was obtained after overnight exposure.

Fig. 4. Effect of pretreatment with iloprost and nicotinamide on the ADP-ribosylation of membranes by pertussis toxin

Details are as in Fig. 3 legend.

in either the absence or the presence of iloprost. Fig. 5 shows the pattern of endogenous ADP-ribosylation of platelet membranes. A 42 kDa protein ($G_s\alpha$) was ADP-ribosylated, and this ribosylation was increased by iloprost. In addition, it was observed that iloprost increases the ADP-ribosylation of several other proteins [10]. This is evident in spite of a darker background in

Fig. 5. SDS/polyacrylamide-gel-electrophoretic analysis of membrane proteins from platelets that have been electroporated and incubated with [32 P]NAD $^{+}$

Samples of electroporated platelets were treated as indicated in the Figure. For other details see the Materials and methods section. The gel autoradiograph was exposed for 7 days.

Fig. 6. SDS/polyacrylamide-gel-electrophoretic analysis of cholera-toxin substrates of membranes from platelets that have been electroporated and incubated during 24 h without or with 10 μ M-iloprost

For other details see the Materials and methods section. The gel autoradiograph was exposed overnight.

lanes 1 and 3 that corresponds to samples not treated with nicotinamide. In control and iloprost-treated platelets, the ADP-ribosylation of the 42 kDa protein was decreased by nicotinamide. This 42 kDa protein co-migrated with $G_s\alpha$ that was ADP-ribosylated by cholera toxin (results not shown).

We also determined whether iloprost changed the ability of the 42 kDa protein to be ADP-ribosylated by cholera toxin after permeabilization. This experiment is shown in Fig. 6. Platelets were electroporated and incubated for 24 h without or with iloprost, and membranes were then prepared and treated with cholera toxin. Fig. 6 shows that pretreatment with iloprost decreased the cholera-toxin-induced ADP-ribosylation of $G_s\alpha$.

[32 P]NAD $^{+}$ can be degraded by NAD glycohydrolases, and hence is not available as a substrate for the ADP-ribosylation. We determined the metabolism of NAD $^{+}$ after 24 h incubation. There were no appreciable differences in the metabolism of [32 P]NAD $^{+}$ to [32 P]ADP-ribose under our various experimental conditions (results not shown). Moreover, after incubation for 24 h NAD $^{+}$ was still present, indicating the absence of NAD glycohydrolases in platelets [10].

DISCUSSION

Prostacyclin analogues such as iloprost produce activation of adenylate cyclase and a parallel loss of agonist-induced platelet responses. Recently it was reported that iloprost and prostaglandin E decreased the cholera-toxin-induced ADP-ribosylation of $G_s\alpha$ in platelets [3]. This was correlated with loss of $G_s\alpha$ from the membrane, which in turn accounted for decreases in fluoride- and guanosine 5'-[β -imidotriphosphate]-dependent adenylate cyclase activation. This decrease in $G_s\alpha$ in the membrane may also explain the heterologous desensitization of adenosine A_2 -receptors by iloprost [3]. However, our information shows that $G_s\alpha$ is not lost from the membrane, but rather its ability to be ADP-ribosylated by cholera toxin changes. In this regard, it is important to mention that ADP-ribosylation factor was identified on the basis of its ability to stimulate cholera-toxin-catalysed ADP-ribosylation of $G_s\alpha$ [15,16]. Therefore, the decrease in ADP-ribosylation of $G_s\alpha$ caused by iloprost could possibly be explained by inhibition of the activity of ADP-ribosylation factor. However, this seems not to be the case, because events that are independent of cholera-toxin-induced ADP-ribosylation are also inhibited. For instance, the ability of guanosine 5'-[β -imidotriphosphate], fluoride and adenosine analogues to activate $G_s\alpha$ and adenylate cyclase in platelets treated with iloprost is impaired [3].

Another covalent modification that could be associated with $G_s\alpha$ is phosphorylation. However, we do not think that the change in $G_s\alpha$ induced by iloprost is related to a cyclic AMP-dependent phosphorylation, since dibutyryl cyclic AMP was unable to mimic the effect of iloprost on the pattern of cholera-toxin-induced ADP-ribosylation.

Endogenous ADP-ribosylation of $G_s\alpha$ has been proposed as an explanation for the autonomous regulation of adenylate cyclase [14]. We propose that a similar reaction might explain the effect of iloprost and, consequently, that iloprost can activate endogenous ADP-ribosylation of $G_s\alpha$. To test this assumption, we

incubated intact platelets with nicotinamide to inhibit endogenous ADP-ribosyltransferase activity [14], and subsequently carried out ADP-ribosylation of the membranes in the presence of cholera toxin. Results showed that $G_s\alpha$ was ADP-ribosylated more efficiently in the membranes obtained from platelets that were treated than in those that were not treated with nicotinamide. ADP-ribosylation of $G_s\alpha$ was decreased when the cells were pretreated with iloprost, and this was partially reversed by pretreatment of the cells with nicotinamide. The most likely explanation of these results is that iloprost causes an increase of platelet NAD:arginine ADP-ribosyltransferase activity. More directly we have assessed the endogenous ADP-ribosylation of $G_s\alpha$ in platelets that had been electroporabilized to allow access of radioactive NAD needed for labelling during ADP-ribosylation. In control conditions a 42 kDa protein ($G_s\alpha$) and other proteins can be ADP-ribosylated and, moreover, iloprost can enhance the ADP-ribosyltransferase activity.

It has been previously proposed that endogenous ADP-ribosylation of $G_s\alpha$ enhances adenylate cyclase activity [14]. However, we have observed that the response of adenylate cyclase to forskolin from platelets treated with iloprost is also impaired (L. Molina y Vedia & E. G. Lapetina, unpublished work). This might suggest that adenylate cyclase is also modified.

The mechanism of heterologous desensitization is a complex process that involves several reactions [3,4,11,17,18]. It is likely that one of those steps is related to increased endogenous ADP-ribosylation of $G_s\alpha$, and that this reaction represents one of the responses to prolonged hormonal stimulation.

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