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Receptor-regulated formation of GTP[γS] with subsequent persistent G_s-protein activation in membranes of human platelets

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Preincubation of human platelet membranes with the ATP analog ATP[γS] led to persistent adenylate cyclase activation. This stimulation was increased by copreincubation with PGE_1 and obliterated by removing endogenous GDP by the NTP-regenerating system, creatine phosphate plus creatine kinase. PGE_1 partially reversed the action of the regenerating system. Control formation of GTP[γS] from ATP[γS] and GDP in platelet membranes was apparently not stimulated by PGE_1. In contrast, in the presence of creatine phosphate plus creatine kinase, which prevented formation of GTP[γS], PGE_1 stimulated formation of this GTP analog, by partially reversing the action of the NTP-regenerating system. The data indicate that GTP[γS] can be formed by a membrane-associated nucleoside diphosphokinase from ATP[γS] and GDP, resulting in persistent G_s-protein activation, and that this process can be stimulated by an agonist-activated receptor.

Nucleoside diphosphokinase; G_s-protein; Adenylate cyclase; GTP analog; Prostaglandin E_1; Platelet membrane

1. INTRODUCTION

The hormone-sensitive adenylate cyclase is regulated by two distinct guanine nucleotide-binding regulatory proteins, G_S and G_i, mediating enzyme stimulation and inhibition, respectively. Activation of these G-proteins following hormone receptor interaction is apparently initiated by the dissociation of GDP bound to the α-subunit of G-proteins and the subsequent binding of GTP to this G-protein component. G-proteins can also be activated by poorly hydrolyzable GTP analogs such as GTP[γS] and guanylyl imidodiphosphate, a process which is time-dependent and which is accelerated by the respective receptor agonists. Due to their resistance to the G-protein GTPase activity, these analogs can cause persistent activation of G-proteins with subsequent persistent stimulation (G_S) or inhibition (G_i) of the adenylate cyclase [1–5].

It has been reported that membrane-associated nucleoside diphosphokinase, catalyzing phosphate group transfer from ATP to GDP, thereby, leading to GTP accumulation, can control the activity of the adenylate cyclase regulatory G-proteins G_S and G_i [6–10]. Furthermore, a close association of nucleoside diphosphokinase with certain G-proteins has been reported, possibly leading, thereby, to a more specific phosphate group transfer to GDP [11–13]. Nucleoside diphosphokinase not only transfers a phosphate group from ATP to GDP, but this enzyme has also been shown to transfer the thiophosphate group from ATP[γS] to GDP leading to the formation of GTP[γS], by using purified enzyme as well as in membranes of HL 60 cells and homogenates of bullfrog atrial myocytes [14–16]. Therefore, we investigated whether such a reaction also occurs in membranes of human platelets and whether it is regulated by agonist-activated receptors. We report

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Abbreviations: ATP[γS], adenosine 5′-*O*-(3-thiotriphosphate); GTP[γS], guanosine 5′-*O*-(3-thiotriphosphate); PGE_1, prostaglandin E_1; G-protein, guanine nucleotide-binding regulatory protein

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here that human platelet membranes possess nucleoside diphosphokinase activity [17], which can catalyze the formation of GTP[γS] from ATP[γS] and GDP, thereby leading to persistent G_S-protein and subsequent adenylate cyclase activation, and that this process can be activated by the adenylate cyclase stimulatory hormone PGE_1.

2. MATERIALS AND METHODS

2.1. *Materials*

Creatine kinase and unlabeled nucleotides were obtained from Boehringer Mannheim (Mannheim); PGE_1 and creatine phosphate from Sigma (Deisenhofen); [8-^{3}H]GDP (13.1 Ci/mmol) from Amersham Buchler (Braunschweig and poly(ethyleneimine)-cellulose F thin-layer chromatography sheets from Merck (Darmstadt). [α-^{32}P]ATP was prepared enzymatically [18].

2.2. *Preincubation of platelet membranes*

Crude membranes of human platelets were prepared as in [19] with 5 mM EDTA present during the membrane preparation procedure. Platelet membranes ($\sim 500\,\mu$g) were incubated for 10 min at 25°C in a total volume of 150 μl. The reaction mixture contained 50 mM triethanolamine-HCl (pH 7.4) and facultative additions such as 50 μM ATP[γS], 2 mM $MgCl_2$, 2 μM PGE_1 or 5 mM creatine phosphate plus 0.4 mg/ml creatine kinase. The reaction was started by addition of ATP[γS] or H_2O and terminated by adding 850 μl ice-cold 10 mM triethanolamine-HCl, (pH 7.4). The membranes were pelleted by centrifugation for 10 min at 30 000 × g. The supernatant was discarded and the membranes were washed twice more with 1 ml ice-cold buffer. The final pellet was resuspended in 10 mM triethanolamine-HCl (pH 7.4) and immediately used for adenylate cyclase assay.

2.3. *Adenylate cyclase assay*

Adenylate cyclase activity was determined in a reaction mixture containing 50 mM triethanolamine-HCl (pH 7.4), 50 μM [α-^{32}P]ATP (0.1 μCi/tube), 2 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM cyclic AMP, 1 mM 3-isobutyl-1-methylxanthine, 1 mM dithiothreitol, 5 mM creatine phosphate and 0.4 mg/ml creatine kinase in a total volume of 100 μl. The reaction was started by adding the preincubated and washed human platelet membranes (40–50 μg protein/tube) and conducted for 10 min at 30°C. Incubation was terminated and cyclic AMP was isolated as in [20].

2.4. *Measurement of GTP[γS] formation*

The assay mixture for measurement of GTP[γS] formation in platelet membranes contained, in a total volume of 50 μl: 0.5 μM [^{3}H]GDP (0.25 μCi/tube), 50 mM triethanolamine-HCl (pH 7.4) and facultative additions used at the same concentrations as described in section 2.2. Reactions were initiated by the addition of human platelet membranes (40–50 μg protein/tube) and conducted for 10 min at 25°C. For termination of the reaction, EDTA was added to give a final concentration of 17 mM in a total volume of 60 μl. The assay tubes were centrifuged for 3 min at 8800 × g and the supernatant was analysed for formation of [^{3}H]GTP[γS] as described [15].

3. RESULTS

When human platelet membranes were preincubated for 10 min at 25°C with the ATP analog ATP[γS] (50 μM), subsequently washed and, then, studied for adenylate cyclase activity without addition of any stimulatory agent, a marked persistent stimulation was observed (fig.1). Without $MgCl_2$ in the preincubation, only weak activation was observed. When $MgCl_2$ (2 mM) was present during preincubation, stimulation by ATP[γS] was increased 4-fold. In the additional presence of GDP (0.3 μM), activation of adenylate cyclase by ATP[γS] was increased further by 40–50%. When the platelet adenylate cyclase stimulatory hormone PGE_1 (2 μM) was included in the preincubation medium and subsequently removed by the membrane washing procedure, the stimulation caused by ATP[γS] in the presence of $MgCl_2$ was increased further by 3–5-fold. In contrast, when the NTP-regenerating system creatine phosphate (5 mM) plus creatine kinase (0.4 mg/ml) was present during the preincubation, no effect of ATP[γS] on subsequently measured adenylate cyclase activity was observed. In the absence of ATP[γS], none of the other additions had a major influence on adenylate cyclase activity, indicating that the membrane washing procedure efficiently removed any free or loosely bound agent, particularly the stimulatory hormone PGE_1. In comparison to ATP[γS], preincubation of platelet membranes with ATP or its analog, adenylyl imidodiphosphate, did not cause adenylate cyclase stimulation (not shown).

These date suggest that ATP[γS] is used by a membrane-associated nucleoside diphosphokinase to form GTP[γS] leading to persistent adenylate cyclase activation. Furthermore, the data obtained with creatine phosphate plus creatine kinase suggested that this treament removed endogenous GDP from the membranes, thus preventing the formation of GTP[γS]. Therefore, we determined whether the stimulatory hormone PGE_1 can counteract this suppressive action of the NTP-regenerating system. As shown in fig.2, creatine phosphate plus creatine kinase completely suppressed the adenylate cyclase stimulatory action of ATP[γS] under control conditions. However, in the additional presence of PGE_1 (2 μM), the inhibitory effect of the NTP-regenerating system

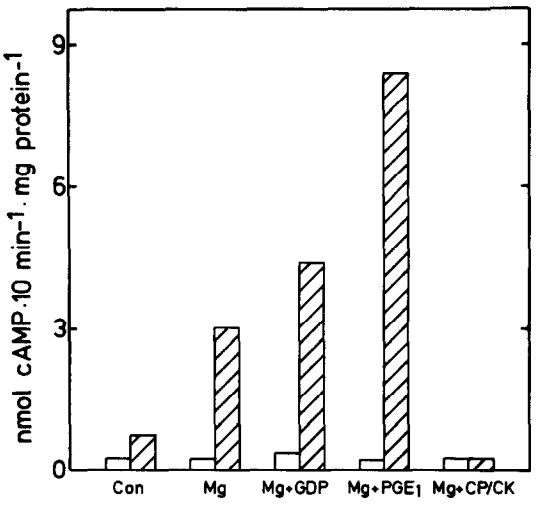

Fig.1. Regulation of ATP[γS]-induced platelet adenylate cyclase stimulation. Human platelet membranes were preincubated for 10 min at 25°C without (open bars) and with 50 μM ATP[γS] (hatched bars) in the absence (Con) and presence of 2 mM $MgCl_2$ (Mg), $MgCl_2$ plus 0.3 μM GDP (Mg + GDP), $MgCl_2$ plus 2 μM PGE_1 (Mg + PGE_1) or $MgCl_2$ plus 5 mM creatine phosphate plus 0.4 mg/ml creatine kinase (Mg + CP/CK). After repeated washing of the membranes, adenylate cyclase activity was determined in the absence of a stimulatory agent, as described in section 2.

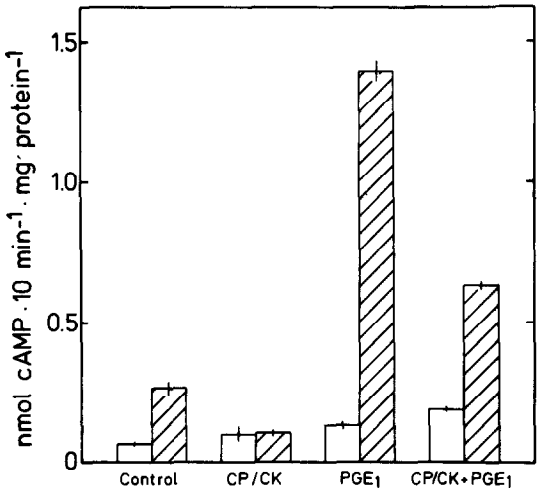

Fig.2. Influence of PGE_1 on ATP[γS]-induced platelet adenylate cyclase stimulation. Human platelet membranes were preincubated for 10 min at 25°C without (open bars) and with 50 μM ATP[γS] (hatched bars) in the absence (Control) and presence of 2 μM PGE_1 (PGE_1), 5 mM creatine phosphate plus 0.4 mg/ml creatine kinase (CP/CK) or a combination of PGE_1 and creatine phosphate plus creatine kinase (CP/CK + PGE_1). $MgCl_2$ (2 mM) was present under each condition. Washing of the membranes and measurement of adenylate cyclase activity were performed as in fig.1. The difference in absolute adenylate cyclase activity between fig.1 and this figure is due to the use of different platelet membrane preparations with different basal and stimulated activities.

was, at least partially, reversed, with ATP[γS] leading to 45% of adenylate cyclase activity compared to that observed in the absence of the NTP-regenerating system.

The data presented so far provided only functional evidence for a nucleoside diphosphokinase-mediated formation of GTP[γS] from ATP[γS] and GDP. Therefore, the formation of GTP[γS] by platelet membranes was measured directly, under the same conditions as used for preincubation of the platelet membranes. As shown in table 1, with 0.5 μM [^{3}H]GDP as acceptor substrate, ATP[γS] (50 μM) led to the formation of 2 pmol [^{3}H]GTP[γS] per mg platelet membrane protein within 10 min at 25°C, corresponding to a GTP[γS] concentration of about 2 nM under the assay conditions used. This value was apparently not affected by the presence of the adenylate cyclase stimulatory hormone PGE_1 (2 μM). As also found in membranes of HL 60 cells [15], the presence of creatine phosphate (5 mM) plus creatine kinase (0.4 mg/ml) almost completely prevented the formation of GTP[γS] from GDP and ATP[γS]. Most interestingly, in the additional presence of PGE_1, which counteracted the suppressive action of the NTP-regenerating system on ATP[γS]-induced adenylate cyclase activation, significant formation of [^{3}H]GTP[γS] was again observed, reaching about 20% of the value observed in the absence of

Table 1

Regulation of GTP[γS] formation in membranes of human platelets

Addition	[^{3}H]GTP[γS] formation (pmol · 10 min^{-1} · mg protein^{-1})	
	Control	PGE_1
None	1.96 ± 0.14	1.96 ± 0.20
CP + CK	0.07 ± 0.02	0.37 ± 0.05

Formation of [^{3}H]GTP[γS] in human platelet membranes (50 μg protein/tube) was measured with 0.5 μM [^{3}H]GDP and 50 μM ATP[γS] as substrates in the absence and presence of 2 μM PGE_1, 5 mM creatine phosphate (CP) plus 0.4 mg/ml creatine kinase (CK) or a combination of PGE_1 and creatine phosphate plus creatine kinase. $MgCl_2$ (2 mM) was present under each condition. Means of triplicate values ± SE are given

the NTP-regenerating system. In the absence of $MgCl_2$ and/or ATP[γS], formation of [^{3}H]GTP-[γS] was not observed (not shown).

4. DISCUSSION

The present data indicate that human platelet membrane G_S-protein can be persistently activated by GTP[γS] locally formed from ATP[γS] and endogenous GDP by the action of a nucleoside diphosphokinase. That the observed adenylate cyclase stimulation is not due to ATP[γS] itself or to a contamination by GTP[γS] was made unlikely by the data obtained with creatine phosphate plus creatine kinase. This NTP-regenerating system, apparently by removing GDP, prevented both the adenylate cyclase stimulation by ATP[γS] and the formation of GTP[γS] from ATP[γS] and GDP. The involvement of a nucleoside diphosphokinase reaction is, furthermore, strengthened by the finding that Mg^{2+}, which is required for nucleoside diphosphokinase activity [21], was necessary for maximal adenylate cyclase activation by ATP[γS], although, on the other hand, the persistent activation of G-proteins by GTP[γS] is also known to require Mg^{2+} [1–5]. Since the stimulatory effect of ATP[γS] was only slightly increased by addition of GDP, it has to be assumed that the membrane preparation used contained enough endogenous GDP serving as acceptor for the nucleoside diphosphokinase-dependent conversion to GTP[γS] in the presence of ATP[γS]. Furthermore, the data obtained with creatine phosphate plus creatine kinase, apparently preventing the formation and subsequent action of GTP[γS], indicate that the endogenous GDP used for the formation of GTP[γS] is not tightly bound to a membrane-associated GDP-binding protein or even only loosely associated with the membrane preparation and, hence, accessible for the creatine kinase.

The enhancing effect of PGE_1 on adenylate cyclase stimulation by ATP[γS] can be explained by either accelerated binding of the formed GTP[γS] to the G_S-protein and/or stimulated formation of GTP[γS] leading to more pronounced G_S-protein activation. In measuring formation of GTP[γS] in the absence of creatine phosphate plus creatine kinase, no enhancing effect of PGE_1 was observed, suggesting that PGE_1 may not stimulate the formation but only the action of the formed GTP[γS]. With the GTP[γS] extraction procedure applied, we may have missed some GTP[γS] formed, i.e., that fraction which is tightly bound to G-proteins and which does not fall off by removing Mg^{2+} with EDTA [5]. However, even by measuring this G-protein-bound GTP[γS], it would not be possible to discriminate between receptor-stimulated formation of GTP[γS] with subsequent binding to the G-protein and unaltered formation but stimulated binding of the GTP analog to the G_S-protein. Therefore, the effect of PGE_1 on GTP[γS] formation was measured in the presence of creatine phospate plus creatine kinase, which completely suppressed control GTP[γS] formation and subsequent G_S-protein activation. In the presence of this NTP-regenerating system, PGE_1 enhanced or partially restored the formation of GTP[γS] from GDP and ATP[γS] as well as the subsequent G_S-protein activation. These data indicate that the agonist-activated PGE_1 receptor can stimulate the formation of GTP[γS]. This agonist-induced stimulation may be due by PGE_1 either providing a GDP pool which is not accessible to creatine kinase or by leading to the formation of a complex of nucleoside diphosphokinase with the G_S-protein [18], liganded with GDP, thereby being accessible to the nucleoside diphosphokinase but not to the creatine kinase. Thus, irrespective of the exact mechanism of action, the data provided herein indicate that the adenylate cyclase stimulatory hormone PGE_1 not only accelerates the binding of GTP[γS] to the G_S-protein, but also that this hormone can increase the formation of GTP[γS] from GDP and ATP[γS]. The PGE_1 stimulation of the GTP[γS]-forming reaction suggests that the activation of G-proteins via the nucleoside diphosphokinase pathway is a receptor-controlled event and, hence, most probably an integrated part of the signal transduction cascade.

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