

Chemical and functional analysis of components of adenylyl cyclase from human platelets treated with phorbol esters

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Human platelets, prelabeled with [32 P]phosphate were treated with tetradecanoylphorbol acetate (TPA) for 5 min at 37°C. Phosphorylation of the components of adenylyl cyclase was determined in membranes using specific antibodies against G-proteins and the catalytic moiety. Less than 0.01 mol of [32 P]phosphate/mol could be detected in immunoprecipitates using antibodies against sequences within the α -subunit of the GTP binding protein G_i . TPA, however, caused the incorporation of 0.67–1.1 mol of [32 P]phosphate per mol of catalyst while 0.13–0.2 mol were found in the absence of TPA. Lack of modification of the α -subunit of G_i was also indicated by the results of reconstitution experiments with purified $G_{i\alpha}$ from bovine brain: adenylyl cyclase in membranes from untreated platelets was significantly more inhibited by added $G_{i\alpha}$, than that from TPA treated cells. While β,γ -subunits were like-wise inhibitory no difference dependent on platelet-pretreatment could be observed.

Adenylyl cyclase; Tetradecanoylphorbol acetate; Phosphorylation; Hormonal inhibition

1. INTRODUCTION

Hormonal inhibition of adenylyl cyclase is mediated by a coupling component, termed G_i , which belongs to the family of GTP-binding proteins exhibiting α,β,γ -configuration [1]. At the moment three different α_i -subunits are known (α_{i1} , α_{i2} , α_{i3}) which, because they can serve as substrates for pertussis toxin, may also be implicated in the regulation of phospholipase C activity [1,2]. The identity of the α_i moiety involved in these two pathways remains to be established, although a recent report does present evidence for α_{i2} being involved in inhibition of adenylyl cyclase [3]. The mechanism of hormonal inhibition is also still a matter of debate, since direct effects (interactions of α_i and/or β,γ -complex with the catalyst) and indirect effects (interference by β,γ -complex with the activation of G_s) have been discussed [4,5].

There is also functional evidence for cross-talk bet-

ween the adenylyl cyclase and the phospholipase C signalling pathways at the level of G_i . Therefore involvement of protein kinase C as a modulator seems to be possible. Attenuation of hormonal inhibition of diacylglycerol and phorbol esters has been recently demonstrated in human platelets [6,7]. In fact, phosphorylation of $G_{i\alpha}$ seemed to be a particularly attractive postulate, since it has been demonstrated that purified G_i from liver could be readily phosphorylated in vitro with an acceptable stoichiometry of 0.5 mol [32 P]phosphate incorporated per mol of $G_{i\alpha}$ [8].

Several groups have tried to demonstrate hormonally induced or phorbol ester-induced phosphorylation of platelet $G_{i\alpha}$ in vivo. Crouch and Lapetina [9] showed [32 P]phosphate-labeling of a 41 kDa protein after immunoprecipitation, but no stoichiometry was reported. However, a recent investigation by Carlson et al. [10] did not reveal [32 P]phosphorylation of $G_{i\alpha}$ after stimulation with TPA analysed by antibodies against two different sequences of $G_{i\alpha}$. Instead, [32 P]phosphate incorporation into a 40 kDa protein was demonstrated which was immunoprecipitated with antibodies designed for specific detection of a novel G_α species termed α_z [11,12]. The functional importance of this 40 kDa protein is unknown. It is not a likely target for attenuation of hormonal inhibition because it lacks the consensus sequence for ADP-ribosylation by pertussis toxin and the hormonal inhibition of adenylyl cyclase is readily neutralized by that toxin [13]. Recently, evidence was presented that α_{i2} was phosphorylated in hepatocytes treated with TPA (and certain hormones) concomitant with loss of Gpp(NH)p-inhibitable adenylyl cyclase activity [14,15].

Abbreviations: TPA, tetradecanoylphorbol acetate; Gpp(NH)p, guanosine 5'-(β,γ -imido)triphosphate; GTP γ S, guanosine 5'-(γ -thio)-triphosphate; C, catalytic component of adenylyl cyclase; G_s , guanine nucleotide binding regulatory component responsible for stimulation of adenylyl cyclase; G_i , guanine nucleotide binding regulatory component responsible for inhibition of adenylyl cyclase, SDS; sodium dodecyl sulfate.

Enzymes: adenylyl cyclase (EC 4.6.1.1), alkaline phosphatase (EC 3.1.3.1), creatine kinase (EC 2.7.3.2).

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It was the aim of this paper therefore to re-examine a possible phosphorylation of coupling proteins but also to extend investigations to the catalytic subunit of the adenylyl cyclase system. Furthermore, the functional interactions of purified subunits of G_i following reconstitution with the phorbol ester modified adenylyl cyclase system of platelets were analysed.

2. MATERIALS AND METHODS

2.1. Materials

Tetradecanoylphorbol acetate (TPA), apyrase, PGE₁, epinephrine, pertussis toxin, cholera toxin, benzethonium chloride and benzamidine were purchased from Sigma (Munich). Gpp(NH)p, GTP- γ S, creatine phosphate, creatine kinase were obtained from Boehringer, Mannheim. Pansorbin (*Staphylococcus aureus* cells) was from Calbiochem and Trasylol from Bayer. All other chemicals were of the highest purity available.

Radiochemicals: Na[¹²⁵I], carrier-free, [α -³²P]ATP (760 Ci/mmol, 1 mCi = 37 MBq), [³²P]orthophosphate carrier-free, [³H]cAMP (15–30 Ci/mmol) were obtained from Amersham Buchler, Braunschweig.

Buffers: RIPA [16]: 150 mM NaCl, 10 mM sodium phosphate, 1% sodium deoxycholate, 0.5% SDS, 1% Nonidet P 40, 1 mM DTE, pH 7.4. Physiological HEPES buffer: 20 mM HEPES, 140 mM NaCl, 2.9 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM glucose, pH 7.4.

2.2. Treatment of platelets with phorbol esters

This was performed essentially according to the method of Williams et al. [7]. Platelet-rich plasma (4- to 5-days-old) obtained from the DRK Würzburg, was centrifuged for 10 min at 160 × g in order to remove any residual erythrocytes. The supernatant was centrifuged at 22°C at 1000 × g for 15 min and the cell pellet washed for 15 min at 1000 × g twice with physiological HEPES buffer (22°C). Platelets were adjusted to ~ 10⁹ cells/ml in the same buffer and incubated for 20 min at 37°C which 0.6 U/ml apyrase and 1 mM aspirin. The cells were centrifuged at 22°C (1000 × g, 15 min) and resuspended at the same density in the above buffer (37°C) containing only apyrase.

Following addition of [³²P]orthophosphate (500 μ Ci/ml) and incubation for 90 min at 37°C, 2 mM creatine phosphate and 0.8 mg/ml creatine kinase were added and the mixture incubated for further 5 min, followed by stimulation with TPA (1 μ M) for 5 min at 37°C. The action of TPA was stopped by the addition of the same volume of ice-cold buffer and centrifugation at 1200 × g for 5 min (4°C). When cells were not prelabeled with [³²P]phosphate, TPA was added directly following a 5 min prewarming period. The pellet was resuspended in 20 vols. of 10 mM sodium pyrophosphate, 10 mM NaF, 5 mM EDTA, 1 mg/ml Trasylol, 1 mM benzamidine, 0.1 mM benzethonium chloride, 0.1 mM sodium metavanadate, 40 μ M phenylmethylsulphonyl fluoride pH 7.4. Lysis was induced by repeated freeze-thawing (liquid nitrogen/waterbath at 30°C). Membranes were washed twice with ice-cold 10 mM triethanolamine, 5 mM EDTA buffer pH 7.4 (30 000 × g, 15 min) and resuspended in the same buffer/10% glycerol at a protein concentration of 5–10 mg/ml. Membranes were stored at –70°C.

2.3. Preparation of antibodies and immunoprecipitation

(a) Antibodies against the adenylyl cyclase catalyst. Preparation of ascites fluid containing monoclonal antibody BBC-4 and its isolation from ascites have been described previously [17]. A covalent Pansorbin-anti-adenylyl cyclase conjugate was prepared according to Schneider et al. [18].

(b) Antisera against peptide sequences of G_s, 46–61 (common to all α subunits except α_2 , serum 6702) and 379–394 (specific for α_s , serum 6712) were obtained after coupling of peptides to serum albumin followed by immunization of New Zealand White rabbits [20].

The efficiency of immunoprecipitation of the catalyst by antibody BBC-4 was determined by quantitative Western blot analysis of SDS-solubilized membranes and immunoprecipitates. Precipitation of α_s and α_i by antisera 6712 and 6702 was quantified using membranes [³²P]ADP-ribosylated with cholera toxin or pertussis toxin. Precipitation of antigens was performed as described in the legends to Figs. 1 and 2. Precipitation efficiencies for catalyst (C), α_s and α_i were 85%, 80% and 48%, respectively. The concentrations of C, α_s and α_i in membranes were determined by quantitative Western blot analysis using the above mentioned antibodies and pure complex consisting of the catalyst and the G_o-subunit (rabbit myocardium for C, [21]), pure G_s (turkey erythrocytes) and pure α_i (bovine brain) as reference preparations. Eight pmol α_s , 30 pmol α_i and 1.7 pmol C per mg of platelet membrane protein were found.

2.4. SDS-polyacrylamide gel electrophoresis and Western blot analysis

For analysis of α -subunits of G-protein, 11% polyacrylamide gels and for analysis of the catalytic subunit of adenylyl cyclase 5–15% gradient gels were used. Transfer of the catalyst to nitrocellulose was in the presence of 0.025% SDS. Antigens for antisera 6702 (α) and 6712 (α_s) were visualized with radio-iodinated protein A, while the catalyst (C) was stained directly with radioiodinated monoclonal antibody BBC-4 [17]. For autoradiography, gels and Western blots were exposed to Kodak XAR 5 films using intensifying screens (Siemens Titan 2 HS). Molecular mass standards (kDa) were myosin (200), β -galactosidase (116), phosphorylase b (97), bovine serum albumin (68), ovalbumin (45) and carbonic anhydrase (30).

2.5. Determination of the stoichiometry of phosphorylation of adenylyl cyclase components

The specific radioactivity of [α -³²P]ATP in platelet lysates was determined according to [22] exploiting the stoichiometric (1:1) autophosphorylation of the regulatory subunit of cAMP-dependent protein kinase from bovine heart. A considerable fraction of the ATP resides in dense granules inside the platelet that is not metabolically labeled and does not participate in cytosolic phosphorylation reactions [23]. Since this ATP pool adds to the cytosolic [³²P]ATP after quenching with perchloric acid [22], a correction of the specific radioactivity had to be made according to the data of Meyers et al. [23]. Therefore the value for the specific radioactivity of ATP was multiplied by a factor of 1.54.

The amount of [³²P]phosphate incorporated into components of the adenylyl cyclase complex was computed as follows:

$$\text{Incorporation (mol/mol)} = \frac{A}{B \times C \times 1.54}$$

A = [³²P] radioactivity in relevant area on polyacrylamide gel (cpm)
B = amount of adenylyl cyclase component applied to gel (pmol)
C = specific radioactivity of ATP pool (cpm × pmol^{–1})

2.6. Miscellaneous procedures

Preparation of G_s from turkey erythrocytes was performed according to [24]. α_i , α_o and β , γ -complex were isolated from bovine brain following published procedures [25–27] with minor modifications. The subspecies composition of α_i (83% α_{i1} and 17% α_{i2}) was determined after [³²P]ADP-ribosylation and separation by polyacrylamide gel electrophoresis in the presence of SDS/urea according to [28].

Adenylyl cyclase activity was measured in 30 mM MOPS, pH 7.4, 15 mM creatine phosphate, 50 μ g/ml creatine kinase, 4.5 mM theophylline, 2 mM MgCl₂, 1 mM dithioerythritol, 2 mg/ml bovine serum albumin, 0.1 mM [³²P]ATP (1–2 × 10⁶ cpm/test tube) for 20 min at 30°C.

Labeling of membrane bound α_s and α_i by [³²P]ADP-ribosylation via cholera toxin or pertussis toxin was performed according to published procedures [29,30].

Protein estimation was performed according to Lowry et al. [31] or according to Schultz et al. [32]. Protein A, molecular mass standards and anti-adenylyl cyclase antibody BBC-4 were radio-iodinated according to Greenwood et al. [33].

3. RESULTS AND DISCUSSION

Short-term treatment of human platelets with TPA leads to attenuation of epinephrine inhibition of several functions of adenylyl cyclase such as stimulation by PGE_1 and forskolin. We can confirm the observations of Johnson et al. [34] with S49 cells that inhibition by GTP-analogs like Gpp(NH)p was also impaired following treatment with phorbol esters. A second consequence of such treatment was enhancement by 30–100% of the enzymatic activity induced by various stimuli, among them exogenous activated G_s , purified from turkey erythrocytes. It should be noted that the amount of G_s to reach half-maximal stimulation was the same before and after TPA treatment. Typical results demonstrating the influence of TPA on stimulation and inhibition of adenylyl cyclase may be seen in Fig. 3.

To examine phosphorylation of the components of the adenylyl cyclase systems we labeled the ATP-pool of intact human platelets with [^{32}P]orthophosphate for 90 min followed by brief treatment with 1 μM TPA. For analysis of $\text{G}_{s\alpha}$ - and $\text{G}_{i\alpha}$ -modification, antibodies against the C-terminal hexadecapeptide of $\text{G}_{s\alpha}$ and against a sequence (46–61) common to α -subunits of G_s , G_i , G_o (but not G_2) was used. This type of antibody also precipitates $\text{G}_{i\alpha}$ -subtypes to a similar extent [35]. A recently described monoclonal antibody, BBC-4, raised against adenylyl cyclase from bovine brain was exploited to monitor the catalytic unit [36]. This antibody was shown to recognize an epitope common to adenylyl cyclases from a variety of tissues and species [21,37]. More recent data have revealed that this antibody binds to a portion of a 21 amino-acid synthetic peptide identical with sequences within the catalyst from bovine brain (Ca^{2+} -sensitive) and rabbit myocard (Ca^{2+} -insensitive) with the exception of one conservative replacement (Arg→Lys) (J. Wallach and S. Mollner, unpublished).

Fig. 1 shows the autoradiographs of SDS gels obtained after immunoprecipitation with anti- G_α antibodies of [^{32}P]phosphate-labeled platelets, with or without subsequent treatment with TPA. As can be seen, neither antibody precipitated substantial amounts of [^{32}P]phosphate-labeled material migrating in the 35–40 kDa range. Less than 0.01 mol [^{32}P]phosphate per mol of α_i and less than 0.01 mol/mol of α_s could be detected, regardless of whether cells had been treated with TPA or not. (The faintly labeled bands at 45 kDa in immunoprecipitates do not represent α_s , which clearly migrates faster.)

It should be noted that the failure to detect [^{32}P]phosphate incorporation into subunits of G-proteins was not a result of interference by possible modifications with the antibody reaction (not shown).

Next we addressed the catalytic moiety of the adenylyl cyclase system. Fig. 2 shows the autoradiograph

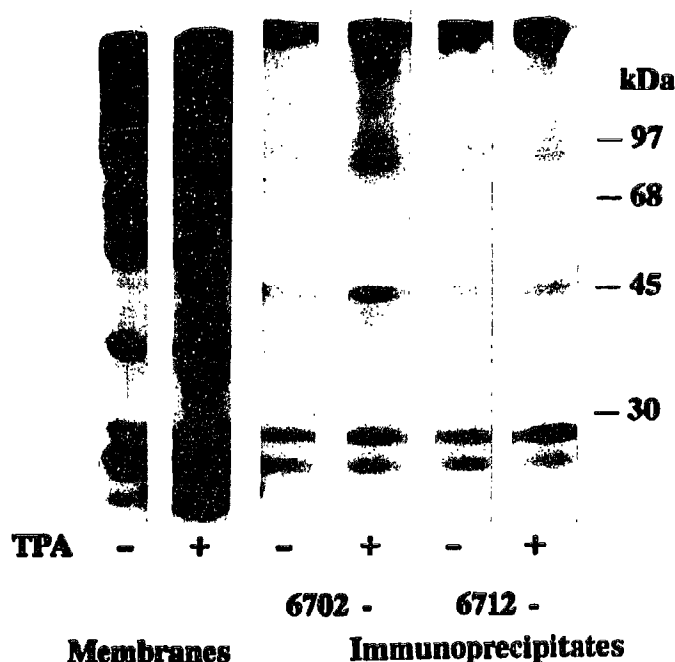


Fig. 1. Immunoprecipitation of [^{32}P]phosphate-labeled platelet proteins by antipeptide antisera 6712 (anti- α_s), and 6702 (anti- α_i). Membranes (3 mg), from [^{32}P]phosphate-labeled platelets, treated with (+) or without (–) TPA were solubilized at 5 mg/ml in RIPA and heated to 100°C for 5 min. 120 μl antisera 6702 or 6712 were added and left for 2 h at 4°C. The mixture was added to 300 μl packed Pansorbin cells and the slurry agitated gently for 1 h at 4°C. After centrifugation (2000 $\times g$) the pellet was washed 4 times with 1.5 ml each of ice-cold RIPA (2000 $\times g$, 5 min). The washed pellet was treated with 250 μl of Laemmli's sample buffer for 5 min at 100°C, and 200 μl of the supernatant were applied to an 11% polyacrylamide gel. Protein trapped by Pansorbin was estimated in parallel using [^{32}P]ADP-ribosylated α_i as tracer. The first two lanes represent RIPA-solubilized [^{32}P]phosphorylated platelet membranes (100 μg). Dried gels were exposed to films for 14 h.

of a 5–15% gradient SDS-polyacrylamide gel of immunoprecipitates obtained with the adenylyl cyclase specific antibody BBC-4, it may be seen that a 150 kDa peptide contained considerably more [^{32}P]phosphate-label if it was derived from platelets previously treated with TPA. As a result a stoichiometry of 0.67–1.1 mol [^{32}P]phosphate per mol of catalytic subunit was obtained for cells that had been treated with TPA. For the catalyst from untreated platelets a stoichiometry of 0.13–0.2 was found. Phosphorylation of the catalyst concomitant with enhancement of adenylyl cyclase activity has also been reported for the enzyme in frog erythrocyte membranes [38].

Absence of modification of α_i via TPA was also indicated after functional reconstitution of pure $\text{GTP}\gamma\text{S}$ -activated α_i with platelet membranes (Fig. 3). Adenylyl cyclase from untreated platelets was considerably more inhibited by α_i than that from treated platelets, irrespective of whether the enzyme was activated via endogenous G_s (PGE_1/GTP) or via exogenous G_s (from

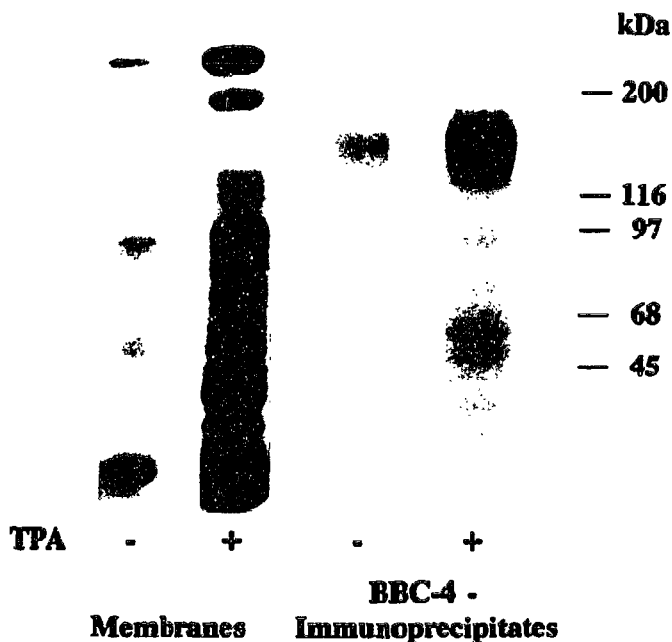


Fig. 2. Immunoprecipitation of [32 P]phosphate-labeled platelet proteins with anti-adenylyl cyclase antibody BBC-4. Membranes, 10 mg, from [32 P]phosphate-labeled platelets treated with (+) and without (-) TPA were solubilized in 2 ml RIPA for 1 h at 22°C. Then 340 μ l of a BBC-4-Pansorbin conjugate (10%) were added, the mixture agitated for 3 h at 22°C and finally spun down at 2000 $\times g$ (5 min). The pellet was washed 5 times with 1 ml ice-cold RIPA. The antigen was removed by treatment of the pellet with 200 μ l of Laemmli's sample buffer for 3 h at 37°C and 180 μ l was applied to a 5-15% polyacrylamide/SDS gel. Protein trapped by Pansorbin was estimated using pure [125 I]-labeled complex consisting of the catalyst and the $G_{s\alpha}$ -subunit from myocardium as tracer. The first two lanes represent RIPA solubilized [32 P]phosphorylated membranes (90 μ g). Dried gels were exposed to films for 4 h.

turkey erythrocytes). Inhibition of membrane-bound adenylyl cyclase by α_i was first reported by Katada et al. [39] and recently also by Kobayashi et al. using G_s -reconstituted S49 cyc $^-$ -membranes [40]. In contrast to the latter authors, we found that inhibition by α_i was mainly of the non-competitive type (H.D. Schulzki, unpublished), suggesting non-identical sites for α_i and α_s on the catalyst. The action of α_i has to be compared to that of the α_2 -adrenergic agonist epinephrine and that of Gpp(NH)p which also affected adenylyl cyclase from TPA-treated and non-treated platelets differently. Likewise, the β, γ -moiety of G-proteins was inhibitory to platelet adenylyl cyclase, as has been shown previously by others [39]. But, when compared to α_i , β, γ -subunits did not discriminate between the control and the TPA treated system. However, phosphorylation of the β -moiety of platelet G-proteins seems to be unlikely, for another reason as well: isoelectric focusing did not reveal a TPA-induced shift in pI compatible with such modification. Attenuation of enzymatic activity was not observed when α_i was not activated by GTP γ S or when α_i was replaced by α_o (not shown).

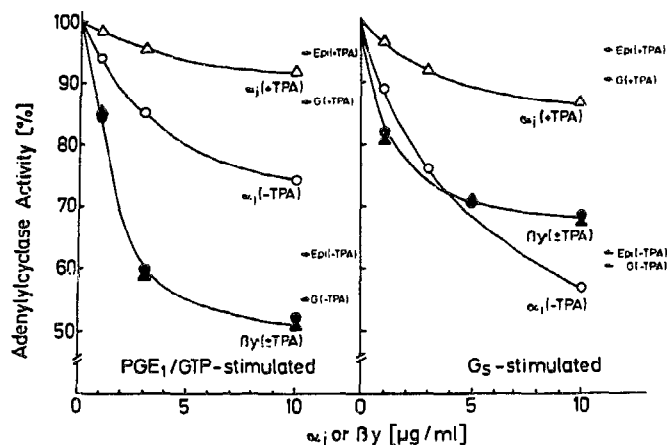


Fig. 3. Inhibition of adenylyl cyclase from TPA treated (+ TPA) and untreated (- TPA) platelets by components of G_i . A. Inhibition of PGE $_1$ -stimulated adenylyl cyclase. Membranes (20 μ g) from untreated (○, ●) and TPA-treated (Δ, ▲) platelets were assayed for adenylyl cyclase activity in the presence of 10 μ M PGE $_1$ and the indicated concentrations of GTP γ S-activated α_i (Δ, ○) or β, γ -subunits (▲, ●) respectively. 100% activity refers to 340 pmol \times mg $^{-1}$ \times min $^{-1}$ (- TPA) and 541 pmol \times mg $^{-1}$ \times min $^{-1}$ (+ TPA). B. Inhibition of G_s -stimulated adenylyl cyclase. Membranes (20 μ g) from untreated (○, ●) and TPA treated (Δ, ▲) platelets were assayed for adenylyl cyclase activity in the presence of 100 ng/ml GTP γ S-activated α_i (Δ, ○) or β, γ -subunits (▲, ●) respectively. 100% activity refers to 160 pmol \times mg $^{-1}$ \times min $^{-1}$ (- TPA) and 232 pmol \times mg $^{-1}$ \times min $^{-1}$ (+ TPA). Unstimulated adenylyl cyclase activity (A and B) was 25 pmol \times mg $^{-1}$ \times min $^{-1}$ in membranes from untreated platelets and 38 pmol \times mg \times min $^{-1}$ in membranes from TPA-treated platelets. Activation of G_i (0.4 μ g/ml) and α_i (41 μ g/ml) was achieved by incubation with 100 μ M and 20 μ M GTP γ S, respectively in 10 mM MgCl $_2$, 0.5 mM Tween 60, 50 mM MOPS buffer, pH 7.4 for 20 min at 30°C. Excessive GTP γ S was removed by centrifugation through dehydrated Sephadex G-25 fine in 50 mM MOPS, pH 7.4, 100 mM NaCl, 0.5 mM Tween 60, 2 mg/ml bovine serum albumin. In order to overcome the effect of any transferred GTP γ S, assays were conducted at high (50 μ M) GTP concentration which by itself did not cause a significant change in adenylyl cyclase activity. For comparison, inhibition by 10 μ M (-) epinephrine (Epi) or 75 μ M Gpp(NH)p (G) is shown (see arrows). The effect of epinephrine was completely blocked by 10 μ M yohimbine. This experiment is representative for 5 independently conducted experiments with different membrane preparations.

The effects of reconstitution with α_i and β, γ -complex presented in Fig. 3 on a percentage basis, suggest that enhancement of enzymatic activity and attenuation of hormonal inhibition are separable events. Although a direct relationship cannot be excluded so far, this would also exclude α_i and the β, γ -complex as targets of TPA-action, because then the relative inhibition should be even greater when these were reconstituted with the TPA-treated system. Attenuation of hormonal inhibition could be explained by modification of the α_2 -adrenoceptor itself. Whilst this may be occurring as well, it can, of course, not explain the failure of GTP-analogs and purified α_i to inhibit platelet adenylyl cyclase. Therefore modification of the catalyst itself, via phosphorylation, as reported here, must be taken

into consideration. This means then that the phosphorylated catalytic moiety must be less effective in interacting with α_i . A definite answer to this question has to await reconstitution experiments employing pure catalyst in its phosphorylated and non-phosphorylated form.

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