Forskolin sensitizes human platelet adenylate cyclase to modulation of substrate (MgATP) affinity by hormones

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Stimulation of human platelet adenylate cyclase by the diterpene forskolin is associated with a decrease in the apparent substrate (MgATP) affinity of the enzyme. Addition of the stimulatory hormone prostaglandin E_1 not only further increased the V_{max} of the forskolin-stimulated platelet adenylate cyclase but also caused a further increase in the K_m value for MgATP, by up to 20-fold compared with basal conditions. On the other hand, the inhibitory hormone adrenaline decreased not only the V_{max} but also the K_m value of the platelet adenylate cyclase stimulated by forskolin, with or without prostaglandin E_1 present. The data indicate that forskolin sensitizes human platelet adenylate cyclase to modulation of substrate (MgATP) affinity by hormones, but there is no such effect in the absence of the diterpene.

INTRODUCTION

The hormone-sensitive adenylate cyclase system is composed of several interacting subunits embedded in the lipid bilayer of the plasma membrane: stimulatory and inhibitory hormone receptors, acting as external discriminators, the adenylate cyclase catalytic moiety, catalysing the formation of cyclic AMP from MgATP, and two guanine nucleotide-binding regulatory components, N_s and N_i , transducing the signal from the hormone-activated stimulatory and inhibitory receptors respectively to the adenylate cyclase (Gilman, 1984; Birnbaumer *et al.*, 1985). Modulation of adenylate cyclase activity by hormone- and guanine nucleotide-activated N_s and N_i is associated with changes in the affinity of the adenylate cyclase for its activating cation Mg^{2+} (Cech *et al.*, 1980; Iyengar & Birnbaumer, 1982; Jakobs *et al.*, 1983; Bockaert *et al.*, 1984). However, a change in the affinity of the adenylate cyclase for its substrate, MgATP, by hormone- and guanine nucleotide-activated N_s and N_i has not been described so far.

By-passing the hormone receptors and probably also the N_s component, the hormone-sensitive adenylate cyclase can be stimulated by the diterpene forskolin (Seamon & Daly, 1981; Daly, 1984). This stimulatory agent can cause a large increase in the V_{max} of the enzyme and can potentiate adenylate cyclase stimulation by hormones. We have previously described that forskolin stimulation of human platelet adenylate cyclase coincides with a decrease in the affinity of the enzyme for its substrate MgATP (Awad *et al.*, 1983). Therefore we were interested in the question whether the forskolin-induced affinity change of the adenylate cyclase for its substrate can be modulated by hormone-activated N_s and N_i . We report here that prostaglandin E_1 (PGE_1) and adrenaline, which respectively increase and decrease basal and forskolin-stimulated activities, reciprocally modulate the K_m value of the platelet

adenylate cyclase for its substrate MgATP, but only in the presence of forskolin.

MATERIALS AND METHODS

Materials

PGE_1 , adrenaline and phosphocreatine were obtained from Sigma. ATP, GTP and creatine kinase were from Boehringer Mannheim. Forskolin was kindly given by Dr. H. Metzger, Hoechst A.G., Frankfurt, Germany. [α - ^{32}P]ATP was prepared enzymically (Walseth & Johnson, 1979) from carrier-free [^{32}P]P $_i$ purchased from New England Nuclear. Membranes of human platelets were prepared as described by Jakobs *et al.* (1982), with 5 mM-EDTA present throughout the membrane preparation procedure.

Adenylate cyclase assay

Adenylate cyclase activity was determined as described by Awad *et al.* (1983), with a reaction mixture containing [α - ^{32}P]ATP (0.2–0.4 μ Ci/tube), $MgCl_2$ and ATP at equimolar concentrations (10–500 μ M), 2 mM excess $MgCl_2$, 0.1 mM-EGTA, 1 mM-3-isobutyl-1-methyl-xanthine, 0.1 mM-cyclic AMP, 1 mM-dithiothreitol, 5 mM-phosphocreatine, 0.4 mg of creatine kinase/ml, 1 mg of bovine serum albumin/ml, 1 μ M-GTP and the additions indicated in 50 mM-triethanolamine/HCl, pH 7.4. The reactions were conducted for 10 min at 37 °C in a total volume of 100 μ l, with 5–15 μ g of platelet membrane protein per tube. Cyclic AMP formed was isolated as described by Jakobs *et al.* (1976). The assays were performed in triplicate, with an intra-assay variation of less than 10% of the means. Fig. 1 shows results of individual experiments, which were repeated six or seven times in a similar manner with different membrane preparations and with different MgATP concentrations

Abbreviations used: N_i and N_s , the guanine nucleotide regulatory proteins that couple inhibitory and stimulatory hormone receptors, respectively, to adenylate cyclase; PGE_1 , prostaglandin E_1 .

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Fig. 1. Double-reciprocal plots of MgATP kinetics of adenylate cyclase activity of human platelet membranes

Adenylate cyclase activity was determined at the indicated concentrations of MgATP without (●; control) and with 30 μM-adrenaline (○; A) as described in the Materials and methods section. (a) Basal activity; (b) activity measured in the presence of 3 μM-PGE₁; (c) activity measured in the presence of 20 μM-forskolin; (d) activity measured in the presence of 20 μM-forskolin plus 3 μM-PGE₁.

(see Table 1). K_m and V_{max} values were estimated from linear double-reciprocal plots of substrate concentration versus enzyme activity.

RESULTS

In the absence of stimulatory and inhibitory ligands, adenylate cyclase of human platelet membranes exhibited a K_m value for MgATP of 15–20 μM (Fig. 1a) (Jakobs *et al.*, 1978; Johnson *et al.*, 1979; Awad *et al.*, 1983). The inhibitory hormone adrenaline (30 μM) decreased basal platelet adenylate cyclase activity by more than 50%. This decrease in activity was not associated with a change

in the K_m value of the platelet adenylate cyclase for its substrate, MgATP. The stimulatory hormone PGE₁ (3 μM) caused a large increase in adenylate cyclase activity (Fig. 1b). This increase in activity was also not associated with a significant change in the K_m value of the enzyme for MgATP. Additionally, adrenaline (30 μM) decreased the PGE₁-stimulated activity by more than 50% without causing a significant change in the K_m value for MgATP. Thus, as reported previously (Jakobs *et al.*, 1978; Johnson *et al.*, 1979), the stimulatory and inhibitory hormones PGE₁ and adrenaline increase and decrease, respectively, the activity of human platelet adenylate cyclase without significant changes in the

Table 1. Influence of hormones on the effect of forskolin on $V_{\max}$ and K_m (MgATP) values of human platelet adenylate cyclase

$V_{\max}$ and K_m (MgATP) values of human platelet adenylate cyclase were measured as described in the Materials and methods section in the presence of 20 μM -forskolin added without or with PGE₁ (3 μM) and adrenaline (30 μM) as indicated. Mean values $\pm$ S.E.M. for the numbers of independent experiments in parentheses are given: * $P < 0.05$ compared with forskolin alone; ** $P < 0.05$ compared with forskolin plus PGE₁.

Addition	K_m (μM)	$V_{\max}$ (nmol of cyclic AMP/min per mg)
Forskolin	125 $\pm$ 5.2 (6)	2.35 $\pm$ 0.21 (6)
Forskolin + adrenaline	77 $\pm$ 6.8 (6)*	1.15 $\pm$ 0.26 (6)*
Forskolin + PGE ₁	349 $\pm$ 60 (7)*	9.18 $\pm$ 2.10 (7)*
Forskolin + PGE ₁ + adrenaline	210 $\pm$ 40 (7)**	4.73 $\pm$ 0.76 (7)**

apparent affinity of the enzyme for its substrate MgATP. From ten similar experiments, a K_m value of 17.3 ± 1.3 (mean $\pm$ S.E.M.) μM for MgATP was estimated.

However, as reported previously (Awad *et al.*, 1983), the increase in platelet adenylate cyclase activity induced by forskolin (20 μM) was associated with a large (about 6-fold) increase in the K_m of the enzyme for the substrate MgATP (Fig. 1c; Table 1). The inhibitory hormone, adrenaline (30 μM) decreased the forskolin-stimulated enzyme activity. The decrease in $V_{\max}$ by adrenaline was similar to that observed when PGE₁ was used as stimulatory ligand. However, in the presence of forskolin, adrenaline decreased the K_m value of adenylate cyclase for MgATP by about 40%, from 125 to 77 μM . When the stimulatory hormone PGE₁ (3 μM) was combined with forskolin (20 μM), the $V_{\max}$ of the platelet adenylate cyclase was further (about 4-fold) increased (Fig. 1d; Table 1). Under this condition, the K_m value of the platelet adenylate cyclase for MgATP was increased to 349 μM , about 20 times the value observed under basal conditions or in the presence of PGE₁ alone. The inhibitory hormone adrenaline (30 μM) decreased the PGE₁+forskolin-stimulated adenylate cyclase activity. Again, as observed with forskolin alone, adrenaline decreased not only the $V_{\max}$ of the PGE₁+forskolin-stimulated enzyme, but also its K_m value for MgATP by about 40%, from 349 to 210 μM .

DISCUSSION

The data presented above show that the apparent affinity of the platelet adenylate cyclase for its substrate MgATP can be controlled by the activity states of the guanine nucleotide-binding regulatory components N_s and N_i. As reported previously (Jakobs *et al.*, 1978; Johnson *et al.*, 1979), the stimulatory and inhibitory hormones PGE₁ and adrenaline increase and decrease, respectively, human platelet adenylate cyclase activity without affecting the apparent affinity of the enzyme for its substrate MgATP. However, in the presence of forskolin, which by itself decreased the platelet enzyme's

affinity for MgATP (Awad *et al.*, 1983), PGE₁ and adrenaline not only induced changes in the $V_{\max}$ of the enzyme but also increased and decreased, respectively, the K_m value of adenylate cyclase for its substrate MgATP. To our knowledge, this is the first example where a hormonal effect on the apparent affinity of adenylate cyclase for its substrate MgATP has been described.

The intriguing question is how these changes in apparent substrate affinity by forskolin and, in the presence of forskolin, by hormone-activated N_s (PGE₁) and N_i (adrenaline) are accomplished. It may be speculated that there are two binding sites for forskolin in the platelet adenylate cyclase system, one at the substrate-binding site, where forskolin may competitively interact with MgATP and lead to the observed increase in the MgATP K_m value, and another allosteric binding site leading to the observed increase in the $V_{\max}$ of the enzyme by forskolin. These two sites may exhibit different sensitivities to forskolin. However, as described previously (Awad *et al.*, 1983), there was no major dissociation between the decrease in apparent substrate affinity of basal adenylate cyclase and the increase in the catalytic activity of the platelet enzyme by forskolin. Furthermore, as shown in above, the observed concomitant increase in both K_m value for MgATP and $V_{\max}$ of the forskolin-stimulated adenylate cyclase by hormone-activated N_s argues a simple competitive interaction of forskolin with MgATP at the substrate binding site. Additionally, hormone-activated N_i decreased not only $V_{\max}$ but also the K_m value for MgATP when forskolin was present. However, without forskolin present, neither hormone-activated N_s nor N_i by themselves significantly affected the apparent MgATP affinity of the platelet adenylate cyclase. Thus it appears that forskolin, by an action at one binding site of the system, induces both activation of the adenylate cyclase and a decrease in the apparent affinity of the enzyme for its substrate MgATP, and that both effects of forskolin are facilitated and hindered by hormone- and guanine nucleotide-activated N_s and N_i respectively. The elucidation of the underlying mechanisms obviously requires studies with resolved components of the system.

The data presented herein should also serve as a cautionary note that, when forskolin is used as a platelet adenylate cyclase-activating agent in combination with stimulatory or inhibitory hormonal agents, the hormonal effects may be masked by concomitant changes in the substrate affinity of the platelet adenylate cyclase. This may explain, e.g., why the platelet adenylate cyclase stimulated by forskolin plus PGE₁, then apparently exhibiting extremely low substrate affinity, seemed to be less sensitive to inhibition by adrenaline than the enzyme stimulated by PGE₁ or forskolin alone when activity was measured at one concentration of MgATP (Insel *et al.*, 1982). On the other hand, the stimulatory effect of forskolin measured on basal adenylate cyclase activity, and particularly in the presence of a stimulatory hormone such as PGE₁, may be masked when using relatively low substrate concentrations.

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