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Inhibition of N_s-Stimulated Human Platelet Adenylate Cyclase by Forskolin

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SUMMARY

The diterpene, forskolin, increases basal adenylate cyclase activity in membranes of human platelets to more than 20-fold with an EC_{50} of about 5 μM. However, when the platelet adenylate cyclase was activated via the stimulatory coupling component, N_s, e.g., by the hormone, prostaglandin E_1, or the stable GTP analog, guanosine 5′-[γ-thio]triphosphate, added in combination with a protease, forskolin was able to inhibit the enzyme. The inhibition was half-maximal and maximal (40–50% inhibition) at 0.01 and 0.1 μM forskolin, respectively, and occurred without apparent lag phase. At a maximally inhibitory concentration, forskolin largely reduced the apparent affinity of the N_s-stimulated platelet adenylate cyclase for its substrate MgATP in a noncompetitive manner, which resulted in a pronounced inhibition by forskolin at low substrate concentrations and a further increase in activity at high MgATP concentrations. Treatment of intact platelets or platelet membranes with agents known to interfere with N_i-mediated adenylate cyclase inhibition did not diminish but even increased the forskolin-induced inhibition of the adenylate cyclase. However, inhibition of the prostaglandin E_1-stimulated adenylate cyclase by forskolin and the inhibitory hormonal agents, thrombin and epinephrine, were not additive at maximally inhibitory concentrations. Furthermore, increasing concentrations of Mg^{2+} and Mn^{2+} reduced (Mg^{2+}) or even reversed (Mn^{2+}) the forskolin-induced inhibition. The data indicate that forskolin apparently has two distinct effects on the platelet adenylate cyclase, namely inhibition and stimulation. The data furthermore suggest that the adenylate cyclase inhibition by forskolin is not mediated by the inhibitory guanine nucleotide-binding protein N_i, but may be due to an action of the diterpene at the adenylate cyclase catalytic moiety, particularly when activated by N_s, or a closely related membrane component.

In the last few years, the diterpene, forskolin, has served as a unique and powerful tool to study the regulation of adenylate cyclase and the role of cyclic AMP in cellular function. Forskolin has been reported to stimulate the hormone-sensitive adenylate cyclase in almost all tissues studied so far (1, 2). The primary site of action of forskolin appears to be the catalytic subunit of the adenylate cyclase system or a closely related membrane component (1–6). Furthermore, forskolin can apparently promote the interaction of hormone and guanine nucleotide-activated N_s, the stimulatory guanine nucleotide-binding regulatory protein, with the catalytic moiety, which results in a potentiation of hormonal stimulation of cyclic AMP formation by forskolin and vice versa in a potentiation of forskolin-induced adenylate cyclase stimulation by stimulatory hormones (1, 2). In binding studies with tritiated forskolin, two binding sites were found, one of high and one of low affinity, with K_D values being in the range of 10^{-8} and 10^{-6} M, respectively (7). The low affinity K_D value corresponds to the EC_{50} value for stimulation of basal adenylate cyclase activity by forskolin, being in the range of 1–10 μM, as shown by studies in various membrane systems (1, 2). The high affinity binding sites were proposed to be associated with the activated complex of catalytic subunit and N_s (7). Indeed, it has recently been reported that stable GTP analogs and the stimulatory hormone, PGE_1, can increase the number of high affinity binding sites in human platelet membranes (8).

In studies on membranes of rat basophilic leukemia cells, we recently observed that forskolin is a weak stimulator of basal adenylate cyclase activity (2-fold stimulation at 100 μM) and that the diterpene at lower concentrations can inhibit the adenylate cyclase stimulated by the stable GTP analog, GTPγS. The inhibition of this adenylate cyclase by forskolin was most pronounced at low substrate (MgATP) concentrations (9). Therefore, we were interested to see whether forskolin can also inhibit adenylate cyclase in a membrane system, where this agent by itself causes a large increase in basal enzyme

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ABBREVIATIONS: N_s, stimulatory guanine nucleotide-binding regulatory protein; PGE_1, prostaglandin E_1; GTPγS, guanosine 5′-[γ-thio]triphosphate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; N_i, inhibiting guanine nucleotide-binding protein.

activity, namely in that of human platelets (10, 11). As shown before (11), the increase in basal platelet adenylate cyclase activity by forskolin is accompanied by a reduction in the affinity of the enzyme for its substrate, MgATP. We report here that forskolin at very low concentrations can inhibit the N_s-stimulated adenylate cyclase of human platelet membranes.

Experimental Procedures

Materials. ATP, GTP, GTPγS, and creatine kinase were purchased from Boehringer Mannheim (Mannheim, FRG). PGE_1, epinephrine, thrombin, and creatine phosphate were from Sigma Chemical Co. (St. Louis, MO). Forskolin was kindly donated by Dr. H. Metzger, Hoechst AG (Frankfurt, FRG) and TPA by Dr. G. Fürstenberger (Deutsches Krebsforschungs-zentrum, Heidelberg, FRG). [α-^{32}P]ATP was prepared enzymatically (12). Crude extracts of bovine sperm particles, containing the protease(s) abolishing actions of the inhibitory guanine nucleotide-binding regulatory component, N_i, on the platelet adenylate cyclase, were prepared as described before (13, 14).

Preparation of platelet membranes. Membranes of control human platelets were prepared as described (15) with 5 mM EDTA present throughout the membrane preparation procedure. In addition, membranes of human platelets pretreated for 2 min with 1 μM TPA were used (16).

Adenylate cyclase assay. Adenylate cyclase activity of human platelet membranes was determined with a reaction mixture containing, if not otherwise indicated, 10 μM [α-^{32}P]ATP (0.3 μCi/tube), 0.2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM cyclic AMP, 1 mM dithiothreitol, 5 mM creatine phosphate, 0.4 mg/ml of creatine kinase, 2 mg/ml of bovine serum albumin, and the additions indicated in 50 mM triethanolamine hydrochloride, pH 7.4, in a total volume of 100 μl. For the kinetic experiments on MgATP affinity, $MgCl_2$ and ATP were added at equimolar concentrations in the presence of a constant concentration of excess $MgCl_2$, by which procedure a close approximate of the true MgATP concentrations is obtained as described by Garbers and Johnson (17). After a 5- or 20-min preincubation at 30° of the membranes (5–10 μg of protein/tube) with the reaction mixture complete except for labeled ATP and forskolin, the measurement of adenylate cyclase activity was initiated by the simultaneous addition of [α-^{32}P]ATP and forskolin or the respective ethanol control solution, which was maximally 0.1%. The reaction was for 10 min at 30°. Cyclic AMP formed was isolated as described (18). Identical activities were obtained when unlabeled ATP was present only during the 10-min incubation period, indicating that ATP was not used up during the preincubation. The extended preincubation (20 min at 30°) was required in order to have linear rates of cyclic AMP formation with time when GTPγS was combined with the sperm protease(s) as shown before (13, 14). The assays were performed in triplicate, differed less than 5% of the means, and were repeated at least twice with similar results as shown herein. Protein was determined according to the method of Lowry *et al.* (19) with human serum albumin as standard.

Results

In the absence of stimulatory ligands, basal adenylate cyclase activity of human platelet membranes is stimulated by forskolin to more than 20-fold, with half-maximal and maximal activation occurring at about 5 and 50 μM, respectively (10, 11). However, when the platelet adenylate cyclase was activated via N_s, forskolin was able to inhibit the enzyme. N_s-mediated stimulation of platelet adenylate cyclase can be accomplished either by a stimulatory hormone such as PGE_1 or by a stable GTP analog such as GTPγS. However, particularly with stable GTP analogs, not only N_s but also N_i is activated (10, 20). Therefore, in order to eliminate the action of GTPγS-activated N_i on the adenylate cyclase and to see only the GTPγS action

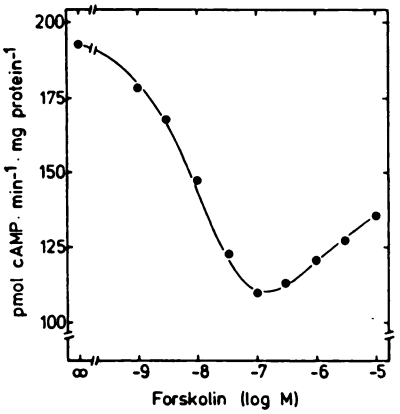

Fig. 1. Influence of forskolin on GTPγS-preactivated human platelet adenylate cyclase. After a 20-min preincubation of the platelet membranes with 1 μM GTPγS and 1 μl/tube of crude sperm protease(s) preparation, adenylate cyclase activity was determined as described in Materials and Methods for 10 min at 30° without and with forskolin at the indicated concentrations.

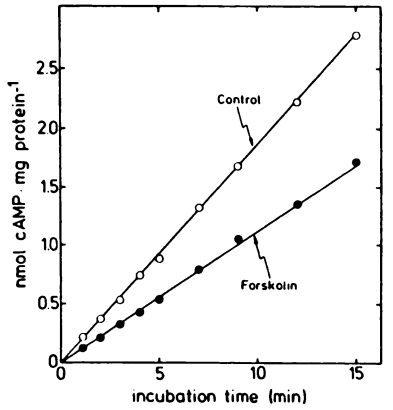

Fig. 2. Time course of forskolin-induced inhibition of human platelet adenylate cyclase. After a 20-min preincubation of the platelet membranes with 1 μM GTPγS and 1 μl/tube of crude sperm protease(s) preparation, measurement of cyclic AMP accumulation was initiated by the addition of labeled ATP without (○) and with (●) 0.1 μM forskolin and continued for the indicated periods of time at 30°.

via N_s, platelet membranes were pretreated with a sperm protease(s) preparation, which has been shown before to abolish hormone- and guanine nucleotide-induced adenylate cyclase inhibition (13, 14). We used this protease(s) preparation instead of trypsin, which can also eliminate N_i-mediated adenylate cyclase inhibition (21), since with trypsin the difference in the concentration range between elimination of N_i action and inactivation of the adenylate cyclase is rather narrow compared to the sperm protease(s) (14, 21). Based on the data obtained in membranes of rat basophilic leukemia cells (9), we first studied whether with a low MgATP concentration (10 μM) as adenylate cyclase substrate the N_s-stimulated platelet enzyme can be inhibited by forskolin and within which concentration range. As shown in Fig. 1, when the platelet adenylate cyclase was preactivated by GTPγS (1 μM) in the presence of a maximally effective concentration of the sperm protease(s) preparation (1 μl/tube), forskolin reduced the stimulated activity in a concentration-dependent manner. Half-maximal and maximal inhibition (40–50%) by forskolin was observed at 0.01 and 0.1 μM, respectively. At concentrations higher than 0.3 μM, forskolin slightly increased the stimulated activity.

Since we used a very low substrate concentration (10 μM), we confirmed that, even after the 20-min preincubation period, the formation of cyclic AMP was linear with time for at least an additional 15-min period at 30° (Fig. 2). Addition of forskolin at a maximally inhibitory concentration (0.1 μM) to the preactivated enzyme caused an inhibition of cyclic AMP formation by about 40%, which occurred without apparent lag phase. There was no change in this reduced activity up to 15 min of incubation. Identical data were obtained when forskolin was present during the 20-min preincubation period (data not shown). The inhibition of the platelet adenylate cyclase by forskolin was dependent on the presence of a stimulatory ligand. In the absence of GTPγS, forskolin (0.1 μM) had a minimal stimulatory effect on adenylate cyclase activity with or without proteases present (Fig. 3). In the presence of the sperm protease(s), GTPγS increased basal adenylate cyclase activity up to 50-fold at 1 μM. Inhibition of the adenylate cyclase by forskolin (0.1 μM) was evident at GTPγS concentrations ≧3 nM. Forskolin flattened the GTPγS concentration response curve without significantly changing the slope of the curve. When GTPγS (1 μM) was used without a protease, adenylate cyclase activity was reduced by a factor of about 10 and forskolin (0.1 μM) caused a small increase in activity by 30–50% (data not shown). Since, in membranes of rat basophilic leukemia cells, forskolin inhibition of adenylate cyclase was most pronounced at low and abolished at high MgATP concentrations (9), we studied whether forskolin affects the MgATP affinity of the N_s-stimulated platelet adenylate cyclase which, as shown before (11), exhibits typical Michaelis-Menten kinetics. In the absence of forskolin, the K_m value of the GTPγS plus sperm protease(s)-stimulated platelet adenylate cyclase was 25 μM (Fig. 4), very similar to the K_m value of the unstimulated enzyme (21 μM) (11). Forskolin, at a maximally inhibitory concentration (0.1 μM), increased the K_m value by about 6-fold to 160 μM. The reduction in apparent substrate affinity of the platelet adenylate cyclase caused by forskolin was obviously not due to a competitive interaction with MgATP. Thus, at low substrate concentrations, forskolin reduced the N_s-stimulated platelet adenylate cyclase activity, whereas at "high" MgATP concentrations (>100 μM) forskolin even increased stimulated activity.

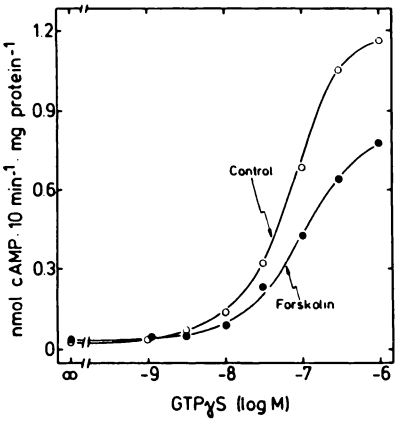

Fig. 3. Inhibition of GTPγS-preactivated platelet adenylate cyclase by forskolin. After a 20-min preincubation of the platelet membranes without and with GTPγS at the indicated concentrations in the presence of 1 μl/tube of crude sperm protease(s) preparation, adenylate cyclase activity was determined without (○) and with (●) 0.1 μM forskolin.

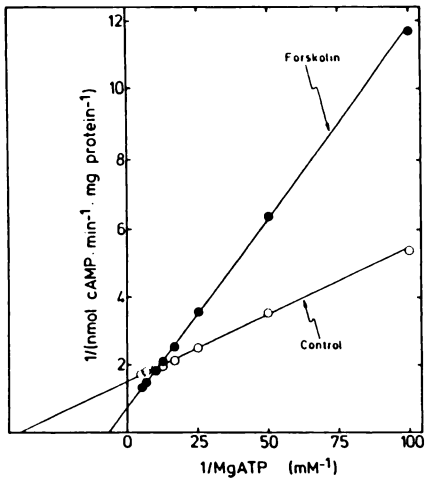

Fig. 4. Influence of forskolin on MgATP kinetics of stimulated human platelet adenylate cyclase. After a 20-min preincubation of the platelet membranes with 1 μM GTPγS and 1 μl/tube of crude sperm protease(s) preparation in the presence of the indicated concentrations of MgATP, adenylate cyclase activity was determined without (○) and with (●) 0.1 μM forskolin. Shown is a double reciprocal plot of enzyme activity versus MgATP concentration.

Several attempts were made to interfere with forskolin-induced inhibition, in order to possibly localize the site of action and to understand the underlying mechanisms. Treatment of platelet membranes with the SH-reagent, *N*-ethylmaleimide, which blocks N_i-mediated adenylate cyclase inhibition (15), did not affect the inhibition of the GTPγS plus sperm protease(s)-preactivated adenylate cyclase by forskolin (Fig. 5). Both control and forskolin-inhibited activities were reduced by *N*-ethylmaleimide in a parallel manner. Even after pretreatment of the membranes with 0.2 mM *N*-ethylmaleimide, which completely eliminates thrombin- and epinephrine-induced inhibition of adenylate cyclase (15, 22), the inhibition by forskolin (0.1 μM) was not decreased (on a percentage basis), whereas the control activity was reduced by more than 60%. However, the divalent cations, Mg^{2+} and Mn^{2+}, largely interfered with the forskolin-induced inhibition. In the absence of forskolin, the GTPγS plus sperm protease(s)-preactivated platelet adenylate cyclase exhibited a high affinity toward Mg^{2+} (13). Similar to inhibitory

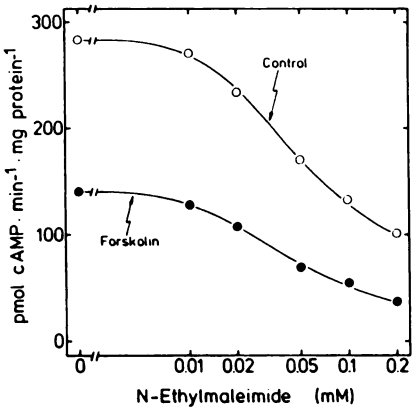

Fig. 5. Influence of *N*-ethylmaleimide on forskolin-induced inhibition of platelet adenylate cyclase. After a 20-min preincubation of the platelet membranes with 1 μM GTPγS and 1 μl/tube of crude sperm protease(s) preparation, the membrane preparation was treated with the indicated concentrations of *N*-ethylmaleimide for 6 min at 30°. After stopping this reaction with 1 mM dithiothreitol, adenylate cyclase activity was determined for 10 min at 30° without (○) and with (●) 0.1 μM forskolin.

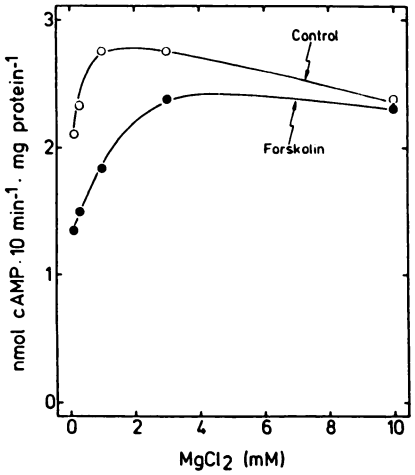

Fig. 6. Influence of Mg^{2+} on forskolin-induced inhibition of human platelet adenylate cyclase. After a 20-min preincubation of the platelet membranes with 1 μM GTPγS and 1 μl/tube of crude sperm protease(s) preparation in the presence of the indicated concentrations of $MgCl_2$, adenylate cyclase activity was determined without (○) and with (●) 0.1 μM forskolin. EGTA was omitted in this experiment.

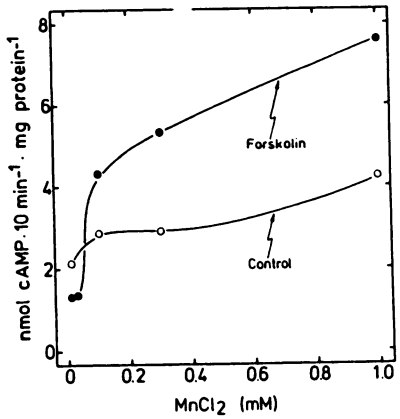

Fig. 7. Influence of Mn^{2+} on forskolin-induced inhibition of human platelet adenylate cyclase. After a 20-min preincubation of the platelet membranes with 1 μM GTPγS and 1 μl/tube of crude sperm protease(s) preparation in the presence of the indicated concentrations of $MnCl_2$, adenylate cyclase activity was determined without (○) and with (●) 0.1 μM forskolin. EGTA was omitted in this experiment.

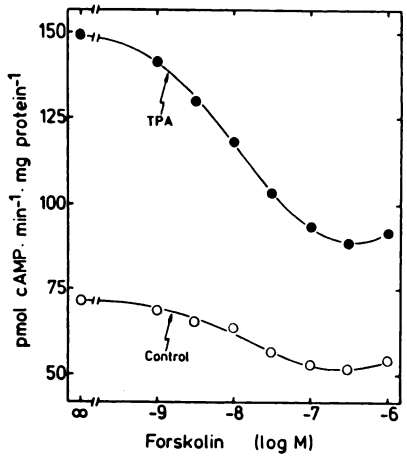

Fig. 8. Inhibition of PGE_1-stimulated platelet adenylate cyclase by forskolin. In membranes of control (○) and TPA (1 μM, 2 min, ●)-pretreated platelets, adenylate cyclase activity was determined after a 5-min preincubation period in the presence of 10 μM PGE_1 and 10 μM GTP without and with forskolin at the indicated concentrations.

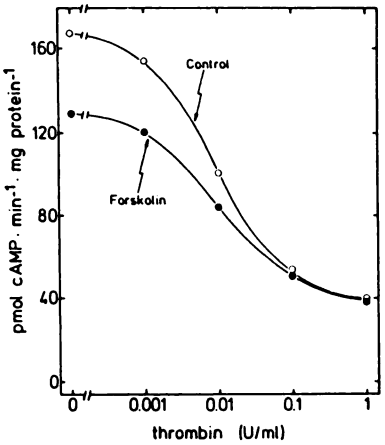

Fig. 9. Interaction of forskolin and thrombin on the PGE_1-stimulated platelet adenylate cyclase. In control platelet membranes, adenylate cyclase activity was determined after a 5-min preincubation period in the presence of 3 μM PGE_1 and 1 μM GTP without (○) and with (●) 0.1 μM forskolin at the indicated concentrations of thrombin.

hormone-induced and N_i-mediated adenylate cyclase inhibition, inhibition of the platelet adenylate cyclase by forskolin (0.1 μM) was accompanied by a reduction in the apparent affinity of the enzyme for Mg^{2+} and a loss of enzyme inhibition at high Mg^{2+} concentrations, as shown in Fig. 6. With Mn^{2+} as activating cation, the adenylate cyclase inhibition by forskolin was abolished at very low concentrations (Fig. 7). At $MnCl_2$ concentrations ≧ 0.1 mM, forskolin (0.1 μM) even caused a further increase in stimulated activity, which was about 2-fold at 1 mM $MnCl_2$.

Forskolin not only reduced the platelet adenylate cyclase activity stimulated by a stable GTP analog, but the PGE_1-stimulated adenylate cyclase was also inhibited by the diterpene at low MgATP concentrations. In control membranes, forskolin decreased the PGE_1 (10 μM) plus GTP (10 μM)-stimulated activity maximally by 25%, within a similar concentration range as observed with the GTPγS-activated enzyme (Fig. 8). In membranes of human platelets pretreated with the phorbol ester, TPA, the PGE_1-stimulated adenylate cyclase activity is increased when measured at high GTP concentrations, e.g., 10 μM (16). In these membranes, the extent of inhibition of the PGE_1 plus GTP-stimulated activity by forskolin was increased (40% inhibition) compared to control membranes. Similar data were obtained when the PGE_1-stimulated adenylate cyclase was studied in the presence of the sperm protease(s) preparation (data not shown). Since the PGE_1-stimulated activity was inhibited by forskolin, we were able to study the interaction of inhibitory hormones with the forskolin-induced inhibition. As shown in Fig. 9, thrombin reduced the PGE_1-stimulated activity maximally by 75%, whereas 0.1 μM forskolin, a maximally inhibitory concentration, caused a 25% reduction in activity. In the presence of both ligands at maximally effective concentrations, there was no indication of an additive type of adenylate cyclase inhibition. When epinephrine was used instead of thrombin as inhibitory hormone, there was also no additive inhibition when studied in the presence of forskolin (data not shown).

Discussion

The data presented in this communication demonstrate that the diterpene, forskolin, which can activate human platelet adenylate cyclase more than 20-fold (10, 11), can also cause inhibition of cyclic AMP formation in platelet membranes. The inhibition and the stimulation of the platelet enzyme by forskolin can be discriminated further by at least two characteristics. First, the stimulation of basal adenylate cyclase activity by forskolin is preceded by a short but significant lag phase, as observed in various membrane systems (1, 2, 23), including human platelet membranes (data not shown). In contrast, inhibition of stimulated adenylate cyclase by forskolin occurred without an apparent lag period. The most striking difference between adenylate cyclase stimulation and inhibition by forskolin is the susceptibility to the diterpene. Whereas half-maximal and maximal activation of basal adenylate cyclase activity has been observed at about 5 and 50 μM, respectively (10, 11), inhibition was half-maximal and maximal at 0.01 and 0.1 μM forskolin, respectively. Thus, the potency of forskolin in inhibiting platelet adenylate cyclase is two to three orders of magnitude higher than its potency in stimulating the enzyme. This difference in potency is reminiscent of the two reported K_D values of the binding sites of forskolin, being in the range of 10^{-8} and 10^{-6} M (7). Whether these sites are physically distinct components or different conformational states of a single membrane component and whether these two sites are associated with adenylate cyclase inhibition and stimulation, respectively, by forskolin requires further studies, particularly with purified forskolin-sensitive components, e.g., the adenylate cyclase catalytic moiety.

Stimulation of adenylate cyclase by forskolin appears to be caused by an action of the diterpene at the catalytic moiety itself or a closely associated membrane component (1–6). This stimulatory action is obviously potentiated by activated N_s. The data presented in this report indicate that the adenylate cyclase inhibition by forskolin is not mediated by the inhibitory guanine nucleotide-binding regulatory component, N_i, but, similar to stimulation, is apparently due to an action of forskolin at the catalytic moiety, particularly when stimulated by activated N_s, or a closely related membrane structure. Treatment of intact platelets or platelet membranes with agents known to reduce or prevent N_i-mediated adenylate cyclase inhibition, such as proteases (13, 21), the SH-reagent, *N*-ethylmaleimide (15), and the phorbol ester, TPA (16), did not diminish adenylate cyclase inhibition by forskolin. In contrast, the use of these agents amplified the inhibitory response to forskolin or were even necessary to observe it at all.

The forskolin-induced adenylate cyclase inhibition is apparently also different from the so-called "P-site" action of adenosine, which is also thought to be due to an action at the catalyst or a closely related component distinct from N_i (24). Both types of adenylate cyclase inhibition, by forskolin and by adenosine, are most pronounced when the enzyme is maximally stimulated. However, the inhibition by adenosine is additive with that of inhibitory hormones in platelet membranes (25), whereas the forskolin-induced inhibition was not. Furthermore, divalent cations (Mg^{2+}, Mn^{2+}) increase the adenylate cyclase inhibition by adenosine (26, 27), whereas forskolin-induced inhibition was impaired (Mg^{2+}) or even reversed into a stimulation (Mn^{2+}) by these divalent cations. In this regard, the forskolin-induced adenylate cyclase inhibition is similar to the N_i-mediated enzyme inhibition, which is also impaired or abolished by Mg^{2+} and Mn^{2+} (28–30). Furthermore, similar to N_i-mediated adenylate cyclase inhibition, forskolin inhibition of human platelet adenylate cyclase was far more sensitive to Mn^{2+} than to Mg^{2+}. The site of action of the divalent cations in preventing adenylate cyclase inhibition by activated N_i or forskolin is apparently the adenylate cyclase itself.

In contrast to all previously reported types of adenylate cyclase inhibition, the forskolin-induced inhibition of the platelet adenylate cyclase was accompanied by a large reduction in the apparent affinity of the enzyme for its substrate, MgATP. This reduction in apparent affinity, which was noncompetitive in nature, resulted in the pronounced inhibition at low substrate concentrations. We have previously reported that forskolin activation of basal platelet adenylate cyclase activity coincides with an increase in the dissociation constant for MgATP, which was about 5-fold at 100 μM forskolin (11). It appears that by the activated N_s the sensitivity of the adenylate cyclase to a forskolin-induced reduction in substrate affinity is increased. As shown herein, after full activation of the platelet enzyme by the stable GTP analog, forskolin, at a very low concentration (0.1 μM), caused about a 6-fold increase in the K_m value of the enzyme for MgATP.

The "physiological" significance of the finding that forskolin can inhibit adenylate cyclase in membranes of human platelets is at present unclear. It has recently been reported that forskolin can cause a decrease in cyclic AMP levels in intact human thyroid cells (31). The inhibition was observed under basal conditions as well as in the presence of a phosphodiesterase inhibitor or the thyroid-stimulating hormone. Most interestingly, similar to what was shown here for platelet membranes, the fall in cyclic AMP levels caused by forskolin occurred at very low concentrations, whereas at higher concentrations forskolin induced the well known increase in cyclic AMP accumulation. These data and those shown herein suggest that forskolin may even inhibit cyclic AMP formation in intact cells. Thus, studies on other membranes and on intact cells are required to elucidate whether the observed inhibitory effect of forskolin on human platelet adenylate cyclase is a general phenomenon of the action of forskolin and whether in intact cells the corresponding fall in cyclic AMP levels with subsequent functional change can be observed.

Acknowledgments

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