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PRELIMINARY COMMUNICATION

SYNERGISTIC INHIBITION OF PLATELET AGGREGATION BY FORSKOLIN PLUS PGE_1 OR 2-FLUOROADENOSINE: EFFECTS OF 2',5'-DIDEOXYADENOSINE AND 5'-METHYLTHIOADENOSINE

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Extracts of Coleus forskohlii have been used in Hindu herbal medicine for many centuries for the treatment of various disorders of cardiovascular, respiratory, gastrointestinal and central nervous systems (1,2). Forskolin (Fig. 1), a diterpene isolated from the roots of these plants (3), has been reported to have a positive inotropic (4), hypotensive (5) and potent stimulatory activity for adenylate cyclases from many tissues (6-12). Recently, forskolin was shown to stimulate adenylate cyclase activity of human platelet membranes (9,13) and to inhibit platelet aggregation (14,15).

We have reported striking potentiation of the inhibition of human platelet aggregation by combinations of forskolin with PGE_1 or 2-fluoroadenosine (F-Ado) (16). Similar results have been described independently by Siegel et al. (15) who also demonstrated synergistic increases in cyclic AMP levels (15).

The present studies examine the effects of an inhibitor of platelet adenylate cyclase, 2',5'-dideoxyadenosine (DDA) (17) and of a presumed antagonist of adenosine receptors, 5'-deoxy, 5'-methylthioadenosine (MTA) (18) on the inhibitions of ADP-induced platelet aggregation caused by forskolin alone or in synergistic combinations with PGE_1 or F-Ado. A preliminary report of these findings has been presented (16).

MATERIALS AND METHODS

Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-labd-14-en-11-one) was purchased from the Calbiochem-Behring Corp., San Diego, CA. MTA and ADP were obtained from the Sigma Chemical Co., St. Louis, MO. DDA was obtained from P-L Biochemicals, Inc., Milwaukee, WI. F-Ado was synthesized by Montgomery and Hewson (19) and provided by the Drug Development Branch, Division of Cancer Treatment of the National Cancer Institute. PGE_1 was obtained from the Upjohn Co., Kalamazoo, MI.

Blood was drawn from normal healthy donors who had not ingested antiplatelet drugs such as aspirin or indomethacin for at least 10 days. Freshly drawn blood was collected in a 0.1 volume of sodium citrate (3.8%). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were separated by a differential centrifugation method (20). Platelet aggregation was measured in PRP (300,000-350,000 platelets/mm^3) by the turbidometric method of Born (21).

RESULTS

Figure 2 shows the effect of forskolin on ADP-induced human platelet aggregation. When PRP was incubated with forskolin (3 μM) for various periods (0-10 min), the degree of inhibition increased with time (20-95%). The inhibition approaches a maximum (90%) in about 5 min. Under these conditions, the IC_{50} of forskolin is 1.7 μM, in contrast to a reported value of 6.0 μM where a preincubation period of only 30 sec was used (15). Forskolin (3 μM), after 5 min of incubation, strongly blocked (95-100%) aggregation induced by arachidonic acid (0.5 mM) and collagen (20 μg/ml). Forskolin also blocked both the primary and secondary phases of epinephrine-stimulated platelet aggregation, with IC_{50} values of 1.2 to 1.7 μM. These results are in basic agreement with other independent findings (15) which show that forskolin is a potent inhibitor of platelet aggregation induced by a wide variety of agents.

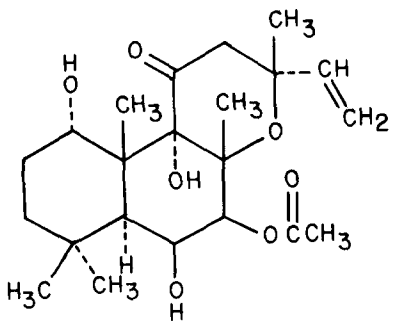

Figure 1. Structure of forskolin.

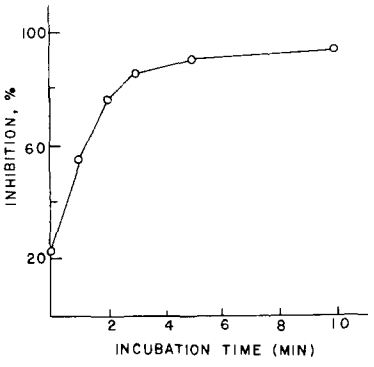

Figure 2. Inhibition of ADP-induced platelet aggregation by forskolin (3 μM) after incubation at 37° with human PRP.

Since forskolin is a potent stimulator of adenylate cyclase from several tissues including human platelets (9,13), its antiplatelet effects were compared to other stimulators of adenylate cyclase such as PGE_1 and F-Ado. Recently, we have shown that the inhibitions of ADP-induced platelet aggregation caused by PGE_1 or Ado and its analogs may be counteracted by DDA, an inhibitor of platelet adenylate cyclase (17). Also, a natural nucleoside, MTA, antagonizes the inhibition caused by Ado and its analogs, but has no effect on the inhibition caused by PGE_1 (17). Table I shows the effects of various concentrations of DDA on the inhibitions caused by forskolin, PGE_1 or F-Ado. DDA did not reverse the inhibitory effects of forskolin, but as reported earlier (17), counteracted PGE_1 and F-Ado. In fact at high concentrations of DDA (200 μM) potentiation of the inhibitory action of forskolin was seen.

Table I. Effects of forskolin, PGE_1 and F-Ado in the presence of DDA on ADP-induced platelet aggregation *

		Platelet Aggregation (%)			
		+ DDA (μM)			
Inhibitors	Concn (μM)	0	60	100	200
Control	--	86	85	87	87
Forskolin	1.5	59	58	60	23
Forskolin	3.0	14	6	6	8
PGE_1	0.7	13	--	72	--
F-Ado	8.0	19	--	69	--

*Human PRP was incubated with forskolin, PGE_1 or F-Ado for 5 min at 37° and ADP (2 or 4 μM) was added to induce aggregation. DDA was added to PRP 1 min before the addition of the inhibitor. These data are representative of three individual experiments performed at different times in which qualitatively similar results were obtained.

As seen in Table II, MTA reversed the inhibitory effects of F-Ado but not of PGE_1, which is consistent with a blockade of the stimulatory (R-site) adenosine receptor of platelet adenylate cyclase. By contrast, MTA potentiated the inhibition of ADP-induced platelet aggregation caused by forskolin.

Table II. Effects of forskolin, PGE_1 and F-Ado in the presence of MTA on ADP-induced platelet aggregation*

Inhibitors	Concn (μM)	Platelet Aggregation (%) + MTA (μM) 0	(50)	(100)
Control	-	86	87	76
Forskolin	0.75	74	36	24
Forskolin	1.5	58	18	3
Forskolin	3.0	14	2	1
PGE_1	0.7	13	-	12
F-Ado	8.0	19	-	77

*Human PRP was incubated with forskolin, PGE_1 or F-Ado for 5 min at 37° and ADP (2 or 4 μM) was added to induce platelet aggregation. MTA was added 1 min before the addition of the inhibitor. These data are representative of three different individual experiments performed at different times in which qualitatively similar results were obtained.

Figure 3 shows the effect of low concentrations (0.3 or 0.6 μM) of forskolin on ADP-induced aggregation in the presence of minimally effective concentrations of PGE_1 (0.11 μM) or F-Ado (2 μM).

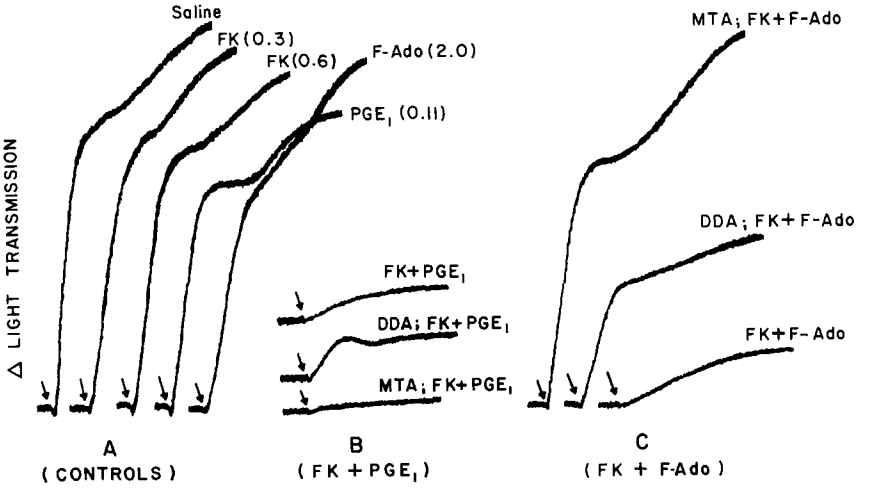

Figure 3. Effects of combinations of suboptimal concentrations of forskolin, PGE_1 or F-Ado in the presence of DDA or MTA on ADP-induced platelet aggregation. Human PRP was incubated at 37° with (A) saline, forskolin, PGE_1 or F-Ado for 5 min; (B) saline, DDA (100 μM) or MTA (100 μM), 1 min then forskolin plus PGE_1, 5 min; and (C) saline, DDA (100 μM) or MTA (100 μM) 1 min then forskolin plus F-Ado, 5 min. After incubation, ADP (4 μM) was added to induce aggregation. Values in the parentheses represent the final concentration (μM) of the agents in the PRP. In combination studies (B) forskolin, 0.6 μM and PGE_1, 0.11 μM; (C) forskolin 0.3 μM and F-Ado, 2 μM.

As reported elsewhere, strong potentiation was seen with both combinations (16). Both DDA and MTA failed to block the synergistic inhibitory effect caused by forskolin plus PGE_1 (Fig. 3,B). However, MTA which does not inhibit platelet adenylate cyclase (17) reversed the potentiated inhibition caused by forskolin plus F-Ado. On the other hand, DDA only partially overcame the inhibition of forskolin plus F-Ado (Fig. 3,C).

DISCUSSION

Forskolin causes time-dependent inhibition of the aggregation of human platelets induced by a wide variety of agents including ADP, collagen, epinephrine and arachidonic acid. Although the results reported here are qualitatively similar to those of Siegel et al. (15), we have found that the IC_{50} of forskolin for ADP-induced aggregation is about 3 to 4 fold lower, i.e. 1.7 μM vs 6.0 μM. Our studies employed preincubation periods of 5 min whereas Siegel et al.(15) used 30 sec. The time study of Fig. 2 demonstrates that about 4-5 min are required to achieve maximal inhibition by forskolin.

Recently, it has been reported that the stimulation of adenylate cyclases from various tissues by forskolin is reversible and does not require a guanine nucleotide (22). It has been suggested that membrane adenylate cyclase consists of two subunits, a catalytic subunit which converts ATP to cyclic AMP, and a regulatory subunit which is guanine nucleotide dependent and responds to hormonal activation (9,22). NaF, GppNHP (guanosine 5'-[β-γ-imido]triphosphate), cholera toxin and hormones apparently stimulate by interaction with the adenylate cyclase regulatory subunit (9). Our results indicate that forskolin acts differently than other stimulators of adenylate cyclase and are consistent with the postulate that it interacts directly with the catalytic subunit of platelet adenylate cyclase as has been proposed for this enzyme from other tissues (9).

Several observations reported here are not readily explained on the basis of earlier findings. DDA, which is believed to interact specifically with the intracellular inhibitory P-site of adenylate cyclase, has been reported to inhibit the stimulation of adenylate cyclase by forskolin (9). As shown in Table I, however, DDA did not reverse the effects of forskolin on platelet aggregation. Also, in agreement with our earlier results (17) MTA blocked the effects of F-Ado, but not of PGE_1 on ADP-induced aggregation (Table II) but, surprisingly, enhanced the inhibitory effects of forskolin in a dose-dependent manner. Another unexplained finding is the complete reversal by MTA of the synergistic effects of forskolin plus F-Ado (Fig. 3,C). Here the synergy between MTA and forskolin (Table II) was not seen. Rather the antagonistic effects of MTA on F-Ado action appear to predominate. Perhaps these effects are mediated through cyclic AMP phosphodiesterase since it has been reported that MTA inhibits this enzyme in mouse lymphocytes and potentiates the actions of cholera toxin and PGE_1 in increasing intracellular cyclic AMP concentrations (23). Further detailed studies of the action of these compounds alone and in combination with forskolin are necessary to evaluate these mechanisms. Studies currently planned include effects on cyclic AMP levels and on platelet adenylate cyclase and cyclic AMP phosphodiesterase. Since this diterpene displays only moderate toxicity (LD_{50} 92 mg/kg i.p. in rats) (24) and acts as a strong inhibitor of platelet aggregation, it may find a future role in the therapy of thromboembolic platelet disorders. As noted above, forskolin-containing plant extracts have been used for many years in humans in India.

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