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Forskolin (a powerful inhibitor of human platelet aggregation)

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Forskolin, a diterpene from the labdane family [1], has been demonstrated to be a powerful stimulant of adenylate cyclase activity in brain and various other tissues [2–5]. We very recently showed that this effect could also be observed in a membrane preparation from human platelets [6]. Forskolin appears to act synergistically with physiological hormonal activators and in an apparently novel manner. From the existing data, it is difficult to conclude definitely whether forskolin acts on the catalytic subunit of adenylate cyclase or needs the presence of a GTP-binding regulatory component [6]. Since the role of cyclic AMP in regulating platelet function is still debated, it was interesting to study the effect of forskolin on platelet aggregation *in vitro* and to correlate the stimulation of adenylate cyclase with the inhibition of aggregation. Because forskolin is a highly hydrophobic compound and may act differently in the two phenomena studied, we used as controls three analogs of forskolin having the same overall structure and hydrophobicity, but devoid of any effect on adenylate cyclase activity.

Materials and methods

EGTA [ethyleneglycolbis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], *l*-epinephrine bitartrate creatine phosphokinase, creatine phosphate, dithiothreitol, ionophore A23287, ADP and ATP were obtained from Sigma Chemical Co.; cyclic AMP was from Calbiochem; EDTA (ethylenediaminetetraacetic acid) was from Merck; [α-^{32}P]ATP (21.5 Ci/mmole) was purchased from New England Nuclear. Cyclic [8-^{3}H]AMP (13 Ci/mmole) was obtained from CEA (Saclay, France); bovine fibrinogen was from Piovet. Forskolin was purchased from Calbiochem or as a gift from Hoechst, France. Compounds I and II, derived from manool, were obtained as a gift from Pr. Fetizon (Ecole Polytechnique, Palaiseau, France). Virescenol B was obtained as a gift from Dr Polonsky (Institut de Chimie des Substances Naturelles, CNRS, Gif sur Yvette, France). See Fig. 1 for the chemical structures of the compounds used.

Preparation of washed human platelets. Blood was collected by vein puncture from young male donors who had taken no drugs for 2 weeks, and drawn into a 60-ml syringe containing 2 ml of EDTA (100 mM). Platelet-rich plasma was prepared by differential centrifugation for 10 min at 500 *g* at room temperature [7]. Platelet-rich plasma was then removed and mixed with an equal volume of washing buffer containing 135 mM NaCl, 13 mM sodium citrate, 5 mM glucose and 1 mM EDTA, buffered at pH 7.5. Platelets were then pelleted by centrifugation for 10 min

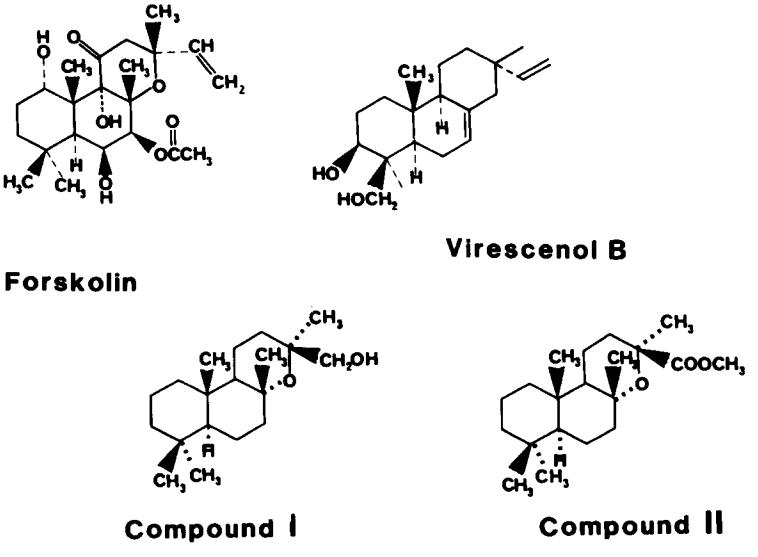

Fig. 1. Chemical structures of forskolin and analogs.

at 600 *g* and resuspended in a buffer containing 135 mM NaCl, 5 mM glucose, 15 mM Tris–HCl and 1 mM EDTA (pH 7.5).

Human platelet membrane preparation. For the study of adenylate cyclase, platelet preparation was obtained by the same procedure, except that the final suspension was made in 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM DTT and 5 mM EGTA [8]. The resulting platelet homogenate was then rapidly frozen in liquid nitrogen and thawed at 20° (operation repeated 3 times). Lysed platelets were then washed by three successive centrifugation steps (30,000 *g*, 20 min) in the same medium. The final pellet was resuspended in the same buffer (but containing 1 mM EGTA) in a suitable volume so as to contain about 0.5–1.5 mg protein/ml. For adenylate cyclase assay, 20 μl of this solution were added to the incubation mixture. Protein concentration was assayed by the method of Bradford [9].

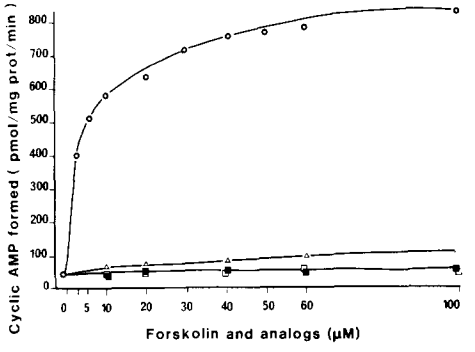

Fig. 2. Dose–response pattern for activation of human platelet adenylate cyclase by forskolin and forskolin analogs. Platelet membranes were incubated for 10 min at 30° with varying concentrations of forskolin (○), compound I (□), compound II (■) and virescenol B (△), and assayed for adenylate cyclase activity as described in *Materials and methods*. The data shown are means of triplicate determinations.

Platelet aggregation measurement. The extent of platelet aggregation was measured by the turbidimetric method of Born [10]. Using a Labintec aggregometer HU176, $CaCl_2$ was added to 500 μl of platelet suspension (adjusted to contained 200,000 platelets/mm^3) to give a final concentration of 1 mM in excess of EDTA. Fibrinogen was then added at a final concentration of 0.35 mg/ml. The rate of aggregation was measured as the tangent to the steepest slope in the light transmittency recordings during the first minute of aggregation and expressed in mm/min.

Adenylate cyclase assay. Adenylate cyclase activity was determined as previously reported [11] with 0.1 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 250 μM EGTA, 1 mM cAMP, 50 mM Tris–HCl (pH 7.6) an ATP-regenerating system (25 mM phosphocreatine and 1 mg/ml of creatine phosphokinase), and the indicated concentrations of forskolin in a final volume of 60 μl. Incubation was initiated by the addition of platelet membranes (10–30 μg protein), and carried out for 10 min at 30°. The reaction was terminated and cyclic AMP measured as previously described [11]. Under these conditions, cyclic AMP formation was linear as a function of time up to 20 min and for platelet protein concentration up to 60 μg/assay. Values represent the means of triplicate determinations and agreed within ±5%. In the experiments designed to test the action of Ca^{2+}, the adenylate cyclase assay was run without EGTA in the incubation medium.

Results and discussion

Concentration dependence of forskolin activation of human platelet membrane adenylate cyclase activity. Forskolin activated platelet membrane adenylate cyclase in a dose-dependent manner with and EC_{50} of about 2.5 μM (Fig. 2); the maximal activation of 17-fold occurred at 100 μM. These data are similar to those previously obtained [6]. Forskolin analogs (I) and (II) had no effect on cyclic AMP synthesis even at 100 μM. Virescenol B was slightly stimulatory with a 2-fold stimulation of adenylate cyclase activity at 100 μM virescenol B.

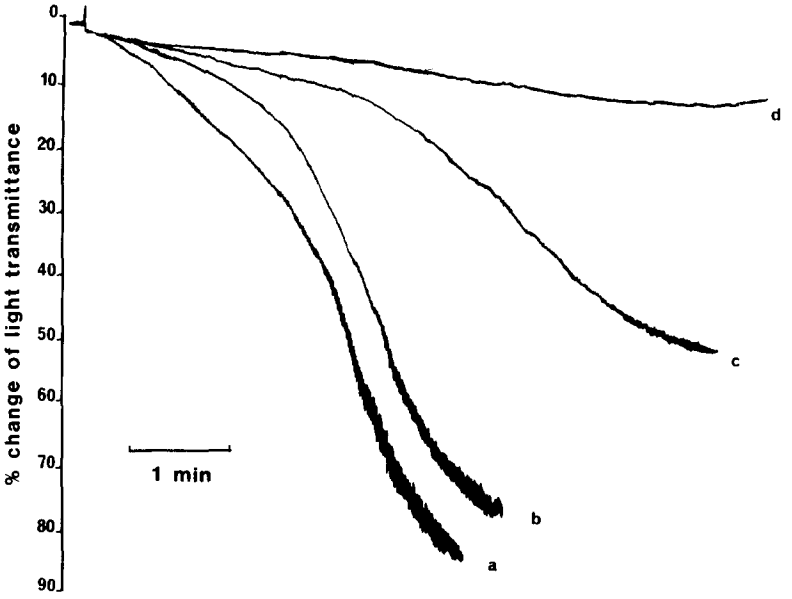

Fig. 3. Effect of forskolin on the aggregation of washed human platelet induced by epinephrine (20 μM). Forskolin was added to the platelet suspension 1 min after incubation in the aggregometer, and epinephrine was added 30 sec thereafter. Curve a was obtained without forskolin; curves b, c and d were obtained with the prior addition of 1, 3 and 10 μM forskolin respectively.

Table 1. Effect of varying concentrations of forskolin on aggregation of washed human platelet induced by ADP, epinephrine and ionophore A23187

Aggregating agent	Forskolin concentration (μM)						
	0	1	2	3	10	100	500
ADP (2 μM)	47	28	25	12	0	nd	nd
Epinephrine (20 μM)	78	80	40	28	0	nd	nd
Ionophore A23187 (20 nM)	82	72	72	72	47	37	0

Aliquots of 500 μl of platelet suspension were incubated in the aggregometer as described in *Materials and methods*. Forskolin was added after 1 min of incubation at 37° and the aggregating agent 30 sec after forskolin addition. Values reported represent the slopes of light transmittency expressed in mm/min.
nd = not determined.

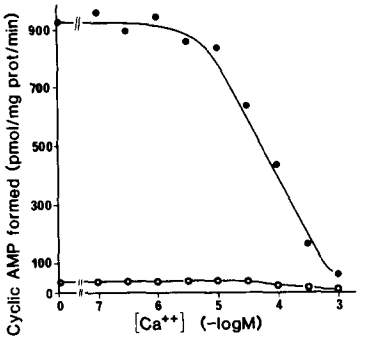

Fig. 4. Dose–response pattern for inhibition of basal and forskolin-activated adenylate cyclase of human platelet. Platelets membranes were incubated for 10 min at 30° in the presence (●) or absence (○) of 10 μM forskolin, with varying concentrations of Ca^{2+}. Adenylate cyclase activity was assayed as described in *Materials and methods* except that the incubation medium contained no EGTA to avoid chelation of Ca^{2+} ions. Data shown are means of triplicate determinations.

Effect of forskolin on aggregation of washed human platelets. Addition of forskolin to a washed platelet suspension markedly reduced the extent of platelet aggregation induced by different agents. Forskolin (3 μM) added 30 sec prior 20 μM epinephrine inhibited the aggregation response to epinephrine by 50% (Fig. 3); complete abolition was obtained with 10 μM forskolin. Addition of the forskolin analogs under the same conditions did not affect the extent of platelet aggregation, even at 100 μM. Table 1 shows results obtained using different aggregating agents. Aggregation of washed platelets induced by ADP was inhibited to the same extent by an identical range of forskolin concentrations; complete abolition occurred both for epinephrine and ADP with 10 μM forskolin. When the calcium ionophore A23187 was used as the aggregating agent, the inhibitory effect of forskolin was less pronounced; 50% inhibition occurred with 10 μM forskolin, and complete inhibition was obtained with 500 μM forskolin. At higher concentrations of ionophore, such as 0.1 μM, which produced a fast and marked aggregation, no inhibitory effect of forskolin was detected, even at high concentrations.

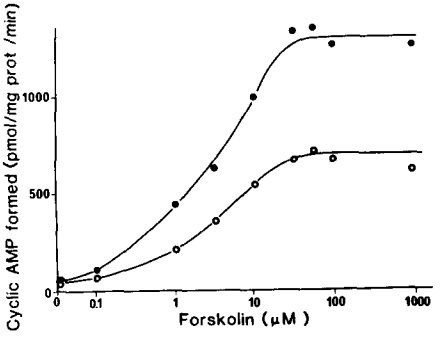

Fig. 5. Effect of Ca^{2+} on the dose–response pattern for activation of human platelet adenylate cyclase by forskolin. Platelet membranes were incubated for 10 min at 30° with varying concentrations of forskolin in the absence (●) or presence (○) of 0.1 μM Ca^{2+}. Adenylate cyclase activity was assayed as in Fig. 4, in the absence of EGTA. Data shown are means of triplicate determinations.

Effect of Ca^{2+} *ions on the forskolin activation of human platelet membrane adenylate cyclase activity.* The lack of an inhibitory effect of forskolin on the aggregation induced by the calcium ionophore could be related to the inhibition of adenylate cyclase activity by the Ca^{2+} ions made available within the cell by the ionophore. As shown in Fig. 4, Ca^{2+} ions, when added to the incubation medium, not only decreased the basal activity of adenylate cyclase, as previously described [12], but also reversed the activatory effect of forskolin. The Ca^{2+} inhibition of the forskolin activation was non-competitive with forskolin (Fig. 5). At 0.1 μM, Ca^{2+} was unable to modify the EC_{50} of the forskolin activation (2.5 μM), while reducing the maximal adenylate cyclase activity by 50%.

Effect of forskolin analogs on aggregation of washed human platelets. The forskolin analogs were tested on platelet aggregation under the same conditions and at concentrations up to 100 μM. Only small and non-reproducible modifications were seen, in part due to the solvent used. Virescenol B, which slightly stimulated the adenylate

cyclase activity, did not induce any substantial change in the extent of platelet aggregation. These compounds are likely to be devoid of any effect on adenylate cyclase as they do not possess OH groups, in the 1 and 9 positions, which have been shown to be necessary for the activation of the adenylate cyclase [13].

It therefore appears that forskolin is a potent inhibitor of platelet aggregation induced by either epinephrine, ADP or ionophore 23187. Two reports have recently also pointed out that forskolin could prevent platelet aggregation in response to ADP, arachidonic acid and collagen [14, 15]. Since forskolin is a very hydrophobic drug, it was reasonable to question the direct marked stimulation brought about by the drug on adenylate cyclase; it could have acted in the aggregatory process by merely altering the physiological properties of the plasma membrane. This hypothesis appeared to be unlikely for two main reasons: (i) Ca^{2+} ions inhibit the effects of forskolin on both adenylate cyclase activity and platelet aggregation and (ii) the three hydrophobic analogs of forskolin tested were inactive both on the adenylate cyclase activity of platelet membranes and on platelet aggregation. These data, therefore, further confirm the role of cyclic AMP in regulating platelet aggregation [16, 17]. Since forskolin is a potentially useful drug clinically because of its inotropic and hypotensive properties [18, 19], it is of interest that it also possesses marked antiaggregating properties.

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Effects of pargyline on *tele*-methylhistamine and histamine in rat brain

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Recent studies of histamine (HA) in brain have focused on the identification and characterization of HA-synthesizing pathways [1], HA receptors [2] and HA metabolites [3–5]. Although HA is metabolized by at least two distinct enzymatic systems in non-neural tissue [6], HA in brain is metabolized almost exclusively by methylation [7, 8].

Tele-Methylhistamine (*t*-MH, see Ref. 9 for nomenclature), the product of HA methylation, has been identified and measured in brain, in other animal tissues, and in human fluids by combined gas chromatography–mass spectrometry (GC–MS) [5, 10–12]. Brain *t*-MH levels may be a sensitive indicator of histaminergic activity, since no uptake mechanism for HA has been discovered, at least not in rat brain [13, 14]. Lesion studies [15, 16] support this hypothesis, implying that HA methylation may occur outside of HA-synthesizing fibers, and suggest that *t*-MH formation may be dependent on neuronal HA release. The similarity in the regional distributions of *t*-MH and HA [4, 5] also suggest that brain *t*-MH levels may be a useful indicator of histaminergic activity.

Several studies have indicated that *t*-MH is metabolized by monoamine oxidase (MAO) in brain. Waldmeier *et al.* [17] showed that type B, but not type A MAO inhibitors increased labeled *t*-MH formation after intracisternal