

# Forskolin Potentiates the Coronary Vasoactivity of Adenosine in the Open-Chest Dog

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**SUMMARY.** Forskolin, a plant diterpene, directly stimulates adenylate cyclase and also potentiates receptor-mediated stimulation of this enzyme by many stimulatory—but not inhibitory—agonists. We exploited the potentiating effect of forskolin to test the hypothesis that adenosine initiates coronary relaxation through activation of adenylate cyclase. In six open-chest dogs, intracoronary forskolin infusions which produced plasma concentrations between 0.15 and 0.48  $\mu\text{M}$  barely changed coronary flow and had no effect on cardiac performance or oxygen metabolism, and did not cause hypotension. Under these conditions, the  $\text{EC}_{50}$  of adenosine, 0.66  $\mu\text{M}$  (range 0.52–0.84), was significantly lower than during control periods before or after forskolin, 1.8  $\mu\text{M}$  (range 1.3–2.4),  $P < 0.05$ . In five of the six dogs, higher doses of forskolin, 0.6–6.3  $\mu\text{M}$ , produced the previously described positive inotropic, chronotropic, and systemic hypotensive effects of this drug. These larger doses of the drug increased coronary flow and  $\text{MVO}_2$  but decreased oxygen extraction, reflecting a combination of direct and metabolic vasodilation. The potentiation of the vasoactivity of adenosine by forskolin supports the hypothesis that the coronary receptor is an adenylate cyclase stimulatory ( $\text{R}_a$  or  $\text{A}_2$ ) receptor. (*Circ Res* 55: 116–119, 1984)

SEVERAL lines of evidence support the hypothesis that the coronary artery adenosine receptor is an adenylate cyclase stimulatory ( $\text{R}_a$  or  $\text{A}_2$ ) receptor. In consonance with the criteria described by Wolff et al. (1981), coronary relaxation by adenosine is mediated by surface receptors (Olsson et al., 1976; Schrader et al., 1977a), is antagonized by theophylline (Afonso, 1970), parallels an increase in arterial cAMP content (Kukovetz et al., 1979), and exhibits greater sensitivity to ethyl adenosine-5'-uronamide (NECA), an adenosine analog selective for  $\text{R}_a$  receptors, than to  $N^6$ -R-1-phenyl-2-propyladenosine (R-PIA), a ligand selective for inhibiting ( $\text{R}_i$  or  $\text{A}_1$ ) receptors (Kusachi et al., 1983). In contrast to its effectiveness in the nanomolar range at  $\text{R}_i$  receptors, adenosine exerts its coronary vasoactivity at micromolar concentrations (Schrader et al., 1977b; Olsson et al., 1979), the operating range characteristic of the low affinity  $\text{R}_a$  receptor.

Forskolin, a diterpene, activates adenylate cyclase directly through a mechanism not associated with receptor interaction and, in low concentrations, acts synergistically to enhance receptor-mediated activation of this enzyme. Available evidence indicates that the potentiating effect of forskolin is specific for receptors that activate adenylate cyclase; this drug apparently does not influence the action of agents that effect receptor-mediated cyclase inhibition (Seamon and Daly, 1981). The ability to amplify the effects of stimulatory agonists, selectively, makes forskolin a useful tool for exploring events subsequent to receptor activation. Indeed, Fredholm et al.

(1983) recently used forskolin to obtain evidence that adenosine acts through  $\text{R}_a$  receptors to stimulate the adenylate cyclase of rat hippocampal slices.

The experiments described here further test the hypothesis that  $\text{R}_a$  receptors mediate coronary relaxation by adenosine, specifically, the prediction that forskolin potentiates the coronary vasoactivity of adenosine.

## Methods

Mongrel dogs weighing  $27 \pm 1$  kg were anesthetized with Na pentobarbital, 30 mg/kg, iv, and maintained throughout an experiment on positive pressure ventilation with  $\text{O}_2$ -enriched air. Adjustments in minute ventilation and, when appropriate, the iv administration of 0.14 M  $\text{NaHCO}_3$  maintained arterial  $\text{PO}_2$ ,  $\text{PCO}_2$ , and pH in the physiological range. Thoracotomy through the left 5th intercostal space exposed the heart for the implantation of an electromagnetic flow probe and plastic occlusive snare near the origin of the left anterior descending coronary artery. We inserted a plastic catheter transmurally into the coronary lumen distal to the snare. A Y-connector linked this coronary catheter to two infusion syringes, thus permitting the simultaneous administration of two solutions at different rates. An electromagnetic flow probe on the aortic root monitored cardiac output and catheters inserted into the aortic root via the left common carotid artery and into the left ventricle via the cardiac apex served for measurement of coronary perfusion pressure and left ventricular pressure. A catheter in the great cardiac vein permitted sampling of the venous drainage of the perfusion field under study.

An earlier publication describes in detail the methods we use to obtain, analyze, and interpret cumulative dose-

response data on the coronary vasoactivity of adenosine and its analogs (Olsson et al., 1979). In the present experiments, we infused a spectrophotometrically standardized solution of adenosine into the coronary artery at a constant rate until the flow response stabilized, then obtained phasic and electronically measured recordings of all blood pressures and flows. These procedures were repeated at successively higher rates of adenosine infusion until a further increase in infusion rate failed to produce any further increase in coronary flow.

We use mean coronary conductance, the quotient of mean coronary flow rate divided by mean aortic root pressure, as an index of coronary tone. Data on infusate concentration and delivery rate, coronary flow rate, and hematocrit yielded an estimate of the adenosine concentration in coronary plasma. Logit transformation of the conductance data and solution of the regression of logit (conductance) on log (adenosine concentration) estimated  $EC_{50}$ , the concentration of adenosine which produced a half-maximum change in coronary conductance.

Blood for estimation of oxygen content was collected anaerobically in syringes containing degassed heparin, and was analyzed galvanometrically (Lex-O-Con TL, Lexington Instruments).

Forskolin can potentiate the coronary vasoactivity of adenosine by two mechanisms other than an action at adenylate cyclase, namely, the inhibition of cardiac adenosine transport and/or adenosine deaminase. We used the method of Oliver and Paterson (1971) to test the effect of forskolin on adenosine uptake by human RBC. The composition of the 1.0-ml assay mixture was: 10  $\mu M$  [ $^{14}C$ ]-adenosine, specific activity, 1.98 TBq/mol; 0.5  $\mu M$  forskolin; 5 mM  $NaH_2PO_4$ , pH 7.4; 0.15 M NaCl, and washed RBC, 1.2%. Tubes containing [ $^{14}C$ ]adenosine but not forskolin served as controls, and tubes to which 20  $\mu M$  NBTGR [6-(4-nitrobenzylthio)guanosine] had been added prior to the addition of RBC accounted for nonmediated transport. Incubation was for 2 minutes at 21°C; preliminary experiments showed  $^{14}C$  uptake was linear over this interval. Centrifugation (Eppendorf model 5414) through dibutyl phthalate separated RBC for liquid scintillation counting. The test of forskolin inhibition of adenosine deaminase employed a spectrophotometric assay (Giusti, 1974), substrate concentrations between 6.9 and 73  $\mu M$  and 4.5 mU enzyme/ml, with or without 0.5  $\mu M$  forskolin. We used the initial rate of the decrease in absorbance at 265 nm to estimate of the rate of deamination.

Forskolin was from Calbiochem, adenosine deaminase (type I) was from Sigma, and [ $8-^{14}C$ ]adenosine was from New England Nuclear. We synthesized NBTGR by heating for 2 hours at 60°C a mixture of 29.9 g (0.1 mol) 2-amino-6-thiopurine riboside, 4.0 g (0.1 mol) NaOH, 21.6 g (0.1 mol)  $\alpha$ -bromo-4-nitrotoluene in 250 ml water. The mixture was cooled, filtered, and the crystalline product washed with water. One recrystallization from ethanol/water yielded 42 g (97%) of product judged pure by analytical high pressure liquid chromatography.

### Experimental Design and Data Analysis

Each experiment consisted of three periods during which we estimated the  $EC_{50}$  of adenosine. During the first and third, which constituted control periods, adenosine was infused into the coronary artery from one syringe and 0.14 M NaCl from the other. During the second experimental period, a solution of 0.1 mM forskolin in 0.14 M NaCl was infused into the second arm of the coronary

catheter at a rate adjusted to produce a barely perceptible rise in coronary flow rate; this rate of forskolin administration was maintained for the remainder of the period. After 15–20 minutes to allow the system to reach a steady state, we assayed the coronary vasoactivity of adenosine.

To ensure that our preparations responded to forskolin in accordance with literature descriptions, we did additional experiments in five of six dogs, testing the hemodynamic responses to larger intracoronary doses. Such observations commenced 30–40 minutes after the end of the second control assay of the coronary vasoactivity of adenosine. During this interval, the coronary infusate was 0.14 M NaCl. Each assessment of the effects of forskolin consisted of recordings of hemodynamic parameters under control conditions and during the steady state responses to intracoronary infusions of forskolin at progressively higher rates. The systemic hypotensive effects of forskolin limited the highest dose to one which produced an approximately half maximum change in coronary flow rate. Samples of arterial and coronary venous blood obtained during the control period and at the highest rate of forskolin administration served for estimates of  $M\dot{V}O_2$ .

Analysis of variance employing the Scheffé test of significance examined the null hypothesis that forskolin would not alter the vasoactivity of adenosine. Differences were considered significant at the 0.05 level. Group data are expressed as mean  $\pm$  SEM.

### Results

The hemodynamic characteristics of all six dogs are listed in Table 1. At the beginning of the experiments, arterial  $P_{O_2}$  averaged  $144 \pm 23$  mm Hg,  $P_{CO_2}$   $37 \pm 3$  mm Hg, and pH  $7.37 \pm 0.01$ ; none of these variables changed significantly during the course of an experiment. Likewise, the hemodynamic parameters did not change significantly between control periods.

Table 1 and Figure 1 shows the effects of a low dose of forskolin on the coronary vasoactivity of adenosine. In all three experimental periods, the maximum dose of adenosine raised coronary conductance over 4-fold but did not change cardiac function or arterial blood pressure. Forskolin infusions which produced an arterial concentration of 0.27  $\mu M$  (range 0.15–0.48  $\mu M$ ) increased cerebral blood flow (CBF) by 13%, changed neither cardiac function nor blood pressure, but slightly though not significantly lowered transcoronary  $O_2$  extraction. Consequently,  $M\dot{V}O_2$  was unchanged. The combination of forskolin administration and a maximally vasodilatory dose of adenosine was likewise without significant effects on cardiac function, blood pressure, or  $M\dot{V}O_2$ , but, as expected, profoundly reduced oxygen extraction. Responses to adenosine during the control periods before and after forskolin were similar.

Figure 1 shows that forskolin significantly enhanced the coronary vasoactivity of adenosine. During the control periods before and after forskolin, the  $EC_{50}$  of adenosine were 1.9  $\mu M$  (range 1.5–2.4  $\mu M$ ) and 1.7  $\mu M$  (range 1.3–2.1  $\mu M$ ), respectively. Forskolin administration lowered the  $EC_{50}$  to 0.66  $\mu M$  (range 0.52–0.84  $\mu M$ ),  $P < 0.05$ .

TABLE 1  
Hemodynamic Effects of Forskolin and Adenosine

Variable	C	A	C	F	A + F	C	A
HR	153 ± 5	151 ± 6	148 ± 6	148 ± 7	153 ± 10	148 ± 11	150 ± 11
BP	100 ± 5	99 ± 6	90 ± 6	98 ± 6	96 ± 6	100 ± 9	99 ± 7
CBF	109 ± 9	500 ± 57*	102 ± 13	115 ± 17	517 ± 65*	108 ± 11	540 ± 56*
CO	1.39 ± 0.30	1.36 ± 0.29	1.27 ± 0.31	1.25 ± 0.29	1.30 ± 0.32	1.24 ± 0.28	1.27 ± 0.29
LVEDP	6 ± 0.7	6 ± 0.6	5 ± 0.4	5 ± 0.4	6 ± 0.5	6 ± 0.3	6 ± 0.4
LV dP/dt	2295 ± 100	2270 ± 105	1995 ± 85	2070 ± 110	2160 ± 120	1900 ± 155	1975 ± 165
MCC	1.11 ± 0.12	5.13 ± 0.47*	1.05 ± 0.14	1.19 ± 0.18	5.39 ± 0.60*	1.06 ± 0.09	5.29 ± 0.64*
MVO <sub>2</sub>			9.01 ± 1.29	9.13 ± 1.28	10.17 ± 2.11		
E O <sub>2</sub>			74 ± 1	66 ± 3	35 ± 6		

Abbreviations: HR, heart rate (beats/min); BP, blood pressure (mm Hg); CBF, coronary flow rate (ml/min per 100 g); CO, cardiac output (liters/min); LVEDP, left ventricular end-diastolic pressure (mm Hg); LV dP/dt, maximum rate of left ventricular pressure rise (mm Hg/sec); MCC, mean coronary conductance (ml/min per 100 g and per mm Hg); MVO<sub>2</sub>, cardiac oxygen consumption rate, (ml O<sub>2</sub>/min per 100 g); E O<sub>2</sub>, transcoronary oxygen extraction (%); C, control; A, maximum adenosine effects; F, forskolin alone; and A + F, adenosine plus forskolin. Asterisks designate means significantly larger than the control for that period. Six dogs, mean coronary artery forskolin concentration, 0.27 μM.

Table 2 summarizes observations in five of the six dogs which demonstrate that higher concentrations of forskolin, mean arterial concentration 1.3 μM, (range 0.6–6.3 μM), produced the positive chronotropic and inotropic effects as well as the systemic hypotensive effects expected of this drug. Forskolin raised MVO<sub>2</sub> significantly, but decreased oxygen extraction, indicating that the increase in coronary flow resulted from a direct effect of the drug on coronary tone in combination with metabolic vasodilation.

Forskolin inhibited neither the rate of [<sup>14</sup>C]adenosine uptake by human RBC nor the catalytic activity of calf intestine adenosine deaminase. The RBC uptake study consisted of eight trials. In RBC suspensions containing 0.5 μM forskolin and 0.1 μM [<sup>14</sup>C]adenosine (ratio of potential inhibitor to per-

meant = 5), the rate of uptake was 43.4 ± 1.5 nmol adenosine/min per ml packed cells, not significantly different from the control value of 37.3 ± 2.6 nmol adenosine/min per ml packed cells. NBTGR-insensitive uptake was negligible, 0.91 ± 0.07 nmol/min per ml packed cells, indicating that, under these conditions, uptake reflects primarily the carrier-mediated transport system (Oliver and Paterson, 1971). At an adenosine concentration of 9.6 μM, the velocity of the adenosine deaminase reaction was 2.41 μmol/min, whereas in an assay containing 6.9 μM adenosine and 0.5 μM forskolin (ratio of potential inhibitor to substrate = 0.07), the rate of deamination was 2.35 μmol/min. A velocity:[substrate] ratio higher in the presence than in the absence of forskolin, 0.34 vs. 0.28 min/liter, argues against forskolin inhibition. At higher adenosine concentrations, i.e., less favorable conditions for demonstrating inhibition, the effect of forskolin was likewise imperceptible.

## Discussion

This experiment exploits the selective effect of forskolin to potentiate the effects of agonists which exert receptor-mediated stimulation—but not inhi-

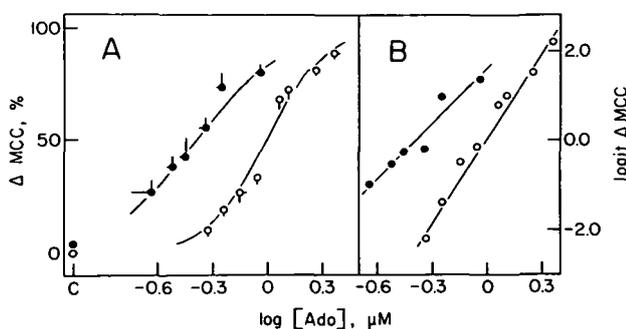


FIGURE 1. Forskolin potentiation of the coronary vasoactivity of adenosine. Summary of observations in all six animals, showing, in panel A, the percentage change in mean coronary conductance, MCC, as a function of log [adenosine]. Open circles describe the dose-response relationship under control conditions; closed circles, that during the intracoronary administration of forskolin. Bars indicate 1 SEM. Panel B is a logit-log transform of the curves shown in panel A. The equation describing the control dose-response relationship is  $\logit(\Delta MCC) = 0.05 + 6.43 (\pm 1.42) \log [\text{adenosine}]$ , and that describing the relationship during forskolin administration is  $\logit(\Delta MCC) = 1.62 + 4.81 (\pm 0.95) \log [\text{adenosine}]$ . The slopes of these curves do not differ significantly.

TABLE 2  
Effects of Intracoronary Forskolin On Systemic Hemodynamics

Variable	Control	During forskolin
HR	143 ± 7	168 ± 5*
BP	101 ± 9	89 ± 12*
CBF	105 ± 5	316 ± 24*
CO	1.07 ± 0.20	1.56 ± 0.43*
LVEDP	6 ± 1	5 ± 1 NS
LV dP/dt	2075 ± 190	3880 ± 815*
MCC	1.07 ± 0.12	3.84 ± 0.72*
MVO <sub>2</sub>	8.35 ± 0.84	15.27 ± 1.69*
E O <sub>2</sub>	69 ± 3	42 ± 3*

Abbreviations are as in Table 1. Five dogs, mean coronary artery forskolin concentration, 1.3 μM.

bition—of adenylate cyclase. In this way, we have obtained further evidence that adenosine initiates coronary relaxation through  $R_a$  receptor-mediated stimulation of adenylate cyclase.

The selection of a dose of forskolin which barely exceeded the threshold for coronary vasodilation was a critical feature of the experimental design. The doses we employed raised coronary flow only slightly and reduced transcoronary oxygen extraction insignificantly. Most importantly,  $MVO_2$  did not change, suggesting that the minor coronary flow change represented a direct vascular effect of the drug. The rate of forskolin administration was constant during the administration of adenosine and, as a result of vasodilation the forskolin concentration, fell further, ultimately to less than 25% of its initial value. Such a progressive dilution of the forskolin concentration probably accounts for the fact that the slope of the adenosine dose-response curve was a third lower during the forskolin administration than during the control periods. The choice of a single dose level of forskolin denied us the opportunity to search for dose-dependent potentiation of adenosine vasodilation. However, this strategy avoided the important effects of forskolin on cardiac metabolism which would have, through metabolic vasodilation, confounded interpretation of the results. Thus, although forskolin caused only a 3-fold reduction in the  $EC_{50}$  of adenosine, we believe this evidence for potentiation is unambiguous.

The ancillary experiments in five of the six dogs confirm the positive chronotropic and inotropic as well as the systemic hypotensive effects of forskolin in the anesthetized dog and augment, in blood-perfused hearts, the observation in isolated, buffer-perfused guinea pig hearts, that forskolin raises  $MVO_2$  and coronary flow rate (Lindner et al., 1978). In the present experiments, forskolin significantly raised  $MVO_2$  but profoundly reduced transcoronary oxygen extraction. This evidence for direct vasodilation in addition to indirect metabolic vasodilation is consistent with other evidence that forskolin is a general smooth muscle relaxant (Muller and Baer, 1983).

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