

FORSKOLIN ACTIVATION OF SEROTONIN-STIMULATED ADENYLATE CYCLASE IN THE LIVER FLUKE *FASCIOLA HEPATICA**

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Abstract—Properties of forskolin activation of adenylate cyclase in the liver fluke *Fasciola hepatica* are described. Forskolin stimulated adenylate cyclase activity in cell-free fluke particles to levels more than 30-fold above the basal rate. This activation was not dependent on guanine nucleotides and, upon washing of the particles, was rapidly reversed. Forskolin potentiated the activation of adenylate cyclase by serotonin (5-HT) and lysergic acid diethylamide (LSD), resulting in both an increase in the maximal level of enzyme activity and a decrease in the apparent activation constant (K_A). The 5-HT antagonist 2-bromo-LSD did not inhibit enzyme activation by forskolin. Furthermore, forskolin had no effect on specific [3 H]LSD binding to fluke particles. Activation of adenylate cyclase by sodium fluoride or guanine nucleotides was modified in a complex manner by forskolin with both stimulatory and inhibitory effects present. The results suggest that forskolin does not interact directly with the 5-HT receptor coupled to adenylate cyclase. Instead, it appears that forskolin effects are, at least in part, due to its ability to alter the interaction between the regulatory and catalytic components of adenylate cyclase. Incubation of intact flukes with forskolin increased their cAMP levels 2- to 3-fold. The concentration dependence of this response was similar to that for forskolin activation of adenylate cyclase in fluke particles, with 300 μ M forskolin giving the maximum response. Forskolin and other agents that increased fluke cAMP levels also stimulated fluke motility.

A serotonin (5-HT†) sensitive adenylate cyclase has been described previously in the liver fluke *Fasciola hepatica* [1-3]. This system is thought to have an important regulatory function in both the carbohydrate metabolism [4] and the motility [2, 5] of the organism. Because of this role, the fluke adenylate cyclase system represents a potentially useful target for chemotherapeutic agents acting against these parasites. Recently, forskolin, a diterpene from the plant *Coleus forskohlii*, was found to be an activator of mammalian adenylate cyclases [6]. We have studied forskolin activation of fluke adenylate cyclase to learn more about the nature of this system and how it relates to what is known about mammalian systems. In this report we describe the properties of forskolin activation of adenylate cyclase in cell-free fluke particles and the ability of forskolin to increase cAMP levels in intact flukes. We also examine the relationship between fluke motility and increased cAMP levels due to forskolin and other agents that stimulate motility.

METHODS AND MATERIALS

Liver fluke particle preparation. Fresh liver flukes

were obtained from bovine liver at a local slaughterhouse and treated as previously described [2]. Cell-free particles were prepared by adding 6 vol. (w/v) of homogenization buffer A (0.33 M sucrose, 5 mM dithiothreitol and 1 mM EDTA) to the flukes and homogenizing the mixture by brief exposure to a "Tekmar Tissumizer". The homogenized suspension was spun at 5000 g for 17 min, and the pellet was resuspended in homogenization buffer B (0.25 M sucrose, 5 mM dithiothreitol, and 1 mM EDTA). The suspension was again spun at 5000 g for 17 min. The final pellet was suspended in homogenization buffer B and used directly, or stored at -70° until just prior to use.

Adenylate cyclase assay. Adenylate cyclase activity in the fluke particles was determined, unless otherwise indicated, by the incubation for 8 min at 30° of a reaction mix containing (final concentrations) 0.1 M sucrose, 50 mM glycylglycine, pH 7.5, 5 mM phosphocreatine, 2 mM $MgCl_2$, 0.1 mM Na-ATP with 0.75 to 1.5 μ Ci [α - 32 P]ATP, 5 units creatine phosphokinase, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) 20 μ M EGTA, 5 mM dithiothreitol, and other compounds as indicated in a final volume of 0.25 ml. The reaction was stopped by the addition of 0.25 ml of a stop solution containing (final concentrations) 2% sodium dodecyl sulfate, pH 7.4, 10 mM EDTA, 10 mM Na-ATP, and 1 mM cAMP. cAMP content was determined by modification of the procedure of Salomon *et al.* [7] as previously described [3]. All values given are the average of duplicate samples.

Measurement of cAMP levels. cAMP levels in intact flukes were determined by a slight modification

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‡ Abbreviations: 5-HT, 5-hydroxytryptamine; serotonin; LSD, *d*-lysergic acid diethylamide; K_A , activation constant; EGTA, ethyleneglycolbis(amino-ethylether) tetra-acetate; GTP γ S, guanosine-5'-O-(3-thio)-triphosphate; and Gpp(NH)p, 5'-guanylyl imidophosphate.

of the radioimmunoassay procedure described by Brooker *et al.* [8]. Intact flukes were incubated at 37° under the indicated conditions after which they were rinsed once with fresh medium and then quickly frozen in Wollenberger clamps chilled in dry ice. The frozen flukes were homogenized in 0.1 N HCl using a motor-driven Teflon pestle. The homogenate was spun at 12,000 g for 20 min, and the supernatant fraction was used to determine cAMP content. All samples and cAMP standards were acetylated by adding 10 μ l of triethylamine followed by 5 μ l of acetic anhydride to 0.5 ml of sample. Fifty microlitres of [125 I]-ScAMP (~10,000 cpm/sample) and 200 μ l of cAMP antiserum were added, and the samples were incubated for 12–16 hr at 4°. After the incubation, 2 ml of isopropyl alcohol was added, and the samples were spun at maximal speed for 20 min in a Beckman TJ-6 table-top centrifuge. The supernatant fractions were aspirated off, and the pellets were counted for 125 I-activity in a Beckman Gamma 5500 counter. cAMP values of the fluke samples were determined by graphical extrapolation from a standard curve obtained for each experiment. The values given are the means of duplicate samples.

[3 H]LSD binding assay. [3 H]LSD binding to the fluke particles was determined using a filtration assay previously described [9]. The incubation conditions were for 5 min at 37°. Specific [3 H]LSD binding was determined by subtracting from the amount of [3 H]LSD bound in a test sample the amount of [3 H]LSD bound from an identical sample containing an excess of unlabeled LSD (100 μ M). All experiments were done in triplicate.

Measurement of fluke motility. The extent of fluke motility was determined by eye on a subjective basis

as previously described [2]. The subjective scale ranged from 1+ (little or no movement) to 4+ (very marked undulations and constant hyperactive movement).

Protein concentrations were determined by the method of Bradford [10] using bovine serum albumin as a standard.

Forskolin stock solution. A stock solution of forskolin (0.01 M) was prepared in 95% ethanol. Consequently, forskolin solutions in reaction mixtures or media used contained a carryover amount of ethanol corresponding to the dilution factor (e.g. 0.095% in the case of 10^{-5} M forskolin).

Materials. Forskolin was purchased from Cal-Biochem. D-Lysergic acid diethylamide was a product of Sandoz, obtained through the National Institute of Mental Health. [3 H]Adenosine 3',5'-cyclic monophosphate (37 Ci/mmol) and [α - 32 P]ATP (400 Ci/mmol) were purchased from Amersham. [3 H]LSD (45–60 Ci/mmol) was a product of New England Nuclear. ATP and guanosine-5'-O-(3-thio)-triphosphate (GTP γ S) were from Boehringer-Mannheim. 5'-Guanylyl imidophosphate (Gpp(NH)p) was from ICN. Adenosine 3',5'-cyclic monophosphate, rabbit skeletal muscle creatine phosphokinase, phosphocreatine, and 5-HT were obtained from Sigma. [125 I]-Succinyl cAMP was provided by Jolanda Schreurs, Stanford. cAMP antibody was a gift from Dr. Gary Brooker, University of Virginia. All other materials were of at least reagent grade and were obtained from various commercial sources.

RESULTS

Forskolin activation of adenylate cyclase in cell-free fluke particles. Adenylate cyclase activity in fluke

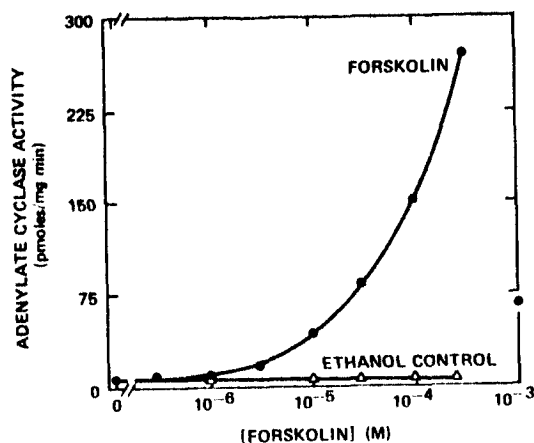


Fig. 1. Forskolin activation of fluke adenylate cyclase. Fluke particles were assayed for adenylate cyclase activity as described in Methods and Materials in the presence of the indicated concentrations of forskolin (●). A stock solution of 0.01 M forskolin was prepared in 95% ethanol. In diluting forskolin to the indicated concentrations, corresponding carryover concentrations of ethanol were obtained in the final reaction mixture (e.g. 0.095% in the case of 10^{-5} M forskolin). The amount of ethanol in the forskolin samples was added alone in the ethanol control (○) to determine its effect on enzyme activity. The values given are means from duplicate samples of a representative experiment.

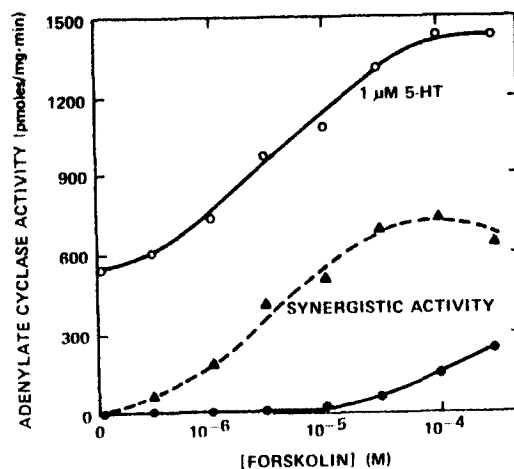


Fig. 2. Synergism of forskolin and 5-HT activation of fluke adenylate cyclase. Fluke particles were assayed for adenylate cyclase activity as described in Methods and Materials in the presence of the indicated concentrations of forskolin with no additions (●) or with 1 μ M 5-HT + 100 μ M GTP (○). The dashed line (▲) represents the synergistic activity and is the amount of activity that is greater than the activity due to the sum of 5-HT and forskolin activation alone. The values given are mean values from duplicate samples of a representative experiment.

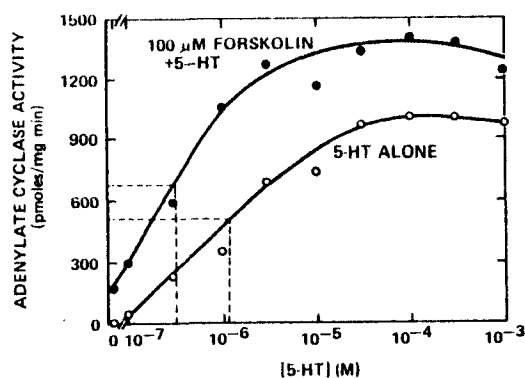


Fig. 3. Effect of forskolin on 5-HT activation of adenylylase. Fluke particles were assayed for adenylylase activity as described in Methods and Materials in the presence of the indicated concentrations of 5-HT and 100 μ M GTP with no additions ($\circ$) or with 100 μ M forskolin ($\bullet$). The values given are means from duplicate samples of a representative experiment.

particles was markedly activated upon the addition of forskolin. Figure 1 shows that forskolin activated adenylylase was concentration dependent. At the maximum concentration that was tested, 300 μ M, forskolin stimulated adenylylase to over thirty-five times the basal enzyme level. Since forskolin was dissolved in 95% ethanol, an ethanol control was run to ensure that the activation was due to forskolin. Concentration of forskolin of 10^{-3} M (with a carry-over of ethanol to give a concentration of 9.5%) activated adenylylase to a lesser extent than did 300 μ M forskolin (Fig. 1). This was probably a result of the higher ethanol concentrations which were found to inhibit basal, 5-HT-stimulated, and NaF-stimulated enzyme activity. Forskolin acti-

vation of adenylylase was not dependent on the addition of GTP and the activation could be rapidly reversed by washing of the particles to remove the forskolin.

The effect of forskolin on 5-HT activation of adenylylase is shown in Fig. 2. It was found that forskolin and 5-HT acted together in a synergistic manner to activate adenylylase to a much greater extent than the sum of the activation of the two compounds alone. Furthermore, as shown in Fig. 3, in the presence of 100 μ M forskolin the ability of 5-HT to activate the enzyme increased with a change in the apparent activation constant (K_A) from 1.2×10^{-6} M to 3.2×10^{-7} M. The synergistic effect of forskolin on activation of adenylylase was even more pronounced when LSD was tested (Fig. 4). LSD, which is a partial agonist in this system, activated adenylylase to 13% of the maximal level obtained with 5-HT activation. However, in the presence of LSD and forskolin the enzyme activity reached 60% of the maximal level obtained with 5-HT and forskolin.

The effects of forskolin on 5-HT- and LSD-stimulated adenylylase activity made it of interest to determine if forskolin was directly interacting at the hormone receptor site. Figure 5 shows that 2-bromo-LSD, a 5-HT antagonist, inhibited both 5-HT-stimulated adenylylase activity and the synergistic response of forskolin plus 5-HT; however, it had no effect in the presence of forskolin alone. The inability of forskolin to interact with the 5-HT receptor was also indicated by binding studies. Previous studies [11] indicated that [3 H]LSD can be used to label these receptors. Forskolin at concentrations up to 100 μ M had no effect on the amount of [3 H]LSD specifically bound to the fluke particles. Forskolin also had no effect on the ability of 5-HT to compete

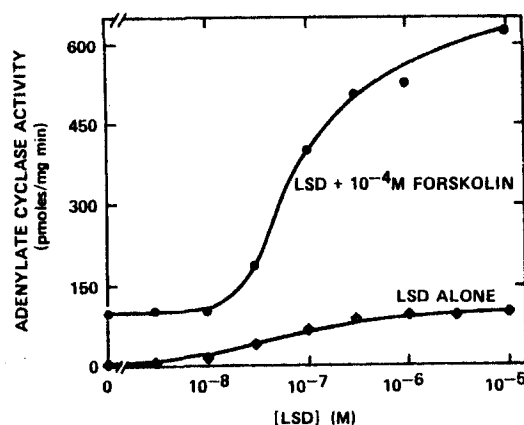


Fig. 4. Effect of forskolin on LSD activation of adenylylase. Fluke particles were assayed for adenylylase activity as described in Methods and Materials in the presence of the indicated concentrations of LSD plus 100 μ M GTP with no additions ($\circ$) or with 100 μ M forskolin ($\bullet$). The values given are means from duplicate samples of a representative experiment. In the same preparation, 100 μ M 5-HT activated adenylylase to a level of 1040 pmoles/mg $\cdot$ min and 100 μ M 5-HT + 100 μ M forskolin activated the enzyme to 1540 pmoles/mg $\cdot$ min.

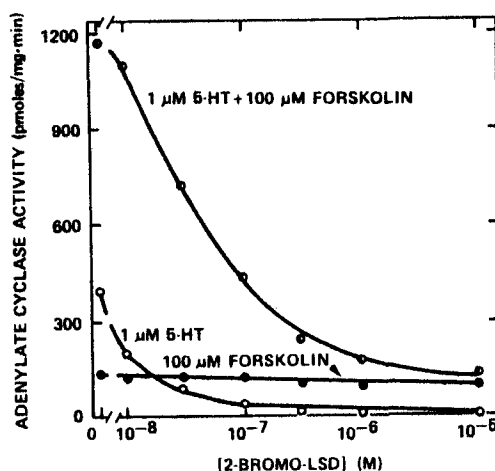


Fig. 5. Effect of 2-bromo-LSD on forskolin activation of adenylylase. Fluke particles were assayed for adenylylase activity as described in Methods and Materials in the presence of the indicated concentrations of 2-bromo-LSD with 1 μ M 5-HT and 100 μ M GTP ($\circ$), with 100 μ M forskolin ($\bullet$), or with 1 μ M 5-HT and 100 μ M GTP plus 100 μ M forskolin ($\bullet$). The values given are means from duplicate samples of a representative experiment. The basal adenylylase activity in this experiment was 6 pmoles/mg $\cdot$ min.

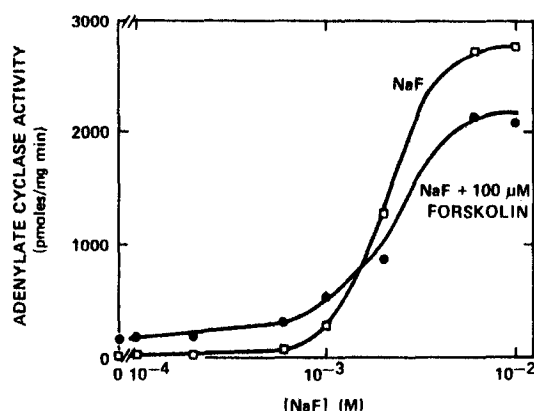


Fig. 6. Effect of forskolin on NaF activation of fluke adenylate cyclase. Fluke particles were assayed for adenylate cyclase activity as described in Methods and Materials in the presence of the indicated concentration of NaF with no additions ($\square$) or with 100 μ M forskolin ($\bullet$). The values given are means from duplicate samples of a representative experiment.

for [3 H]LSD binding sites (data not shown). Thus, both the adenylate cyclase activity data and the [3 H]-LSD binding data indicate that forskolin activation of adenylate cyclase was not mediated by any direct interaction with fluke 5-HT receptors.

The effect of forskolin on adenylate cyclase activation mediated through the regulatory component of the system is complex. Figure 6 shows the effect of forskolin on sodium fluoride activation of adenylate cyclase. At low sodium fluoride concentrations, the addition of forskolin increased adenylate cyclase activity to levels which were slightly more than additive. At high sodium fluoride concentrations, however, addition of forskolin decreased enzyme activity. Forskolin also affected the activation of adenylate cyclase by guanine nucleotides. Table 1 shows that activation of adenylate cyclase by

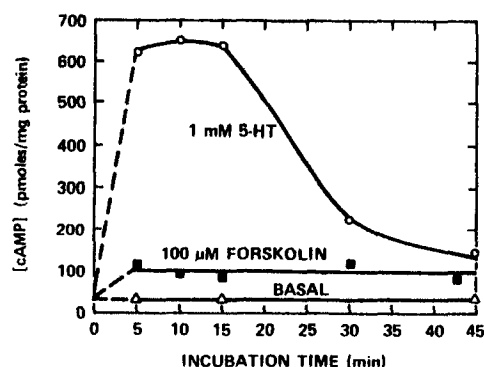


Fig. 7. Effect of forskolin on cAMP levels in intact flukes. Liver flukes were incubated at 37° in media containing no additions (Δ), 100 μ M forskolin ($\blacksquare$), or 1 mM 5-HT ($\circ$). At the indicated incubation times, the cAMP content from an aliquot of flukes was determined by radioimmunoassay as described in Methods and Materials. The results are mean values from duplicate samples of a single experiment.

Gpp(NH)p and forskolin was nearly additive. However, activation by GTP γ S was inhibited by forskolin. These results support previous experiments [12].

Effect of forskolin on cAMP levels in intact flukes. Incubation of intact flukes with 100 μ M forskolin increased their cAMP level to more than twice the level found in control flukes. Figure 7 shows that the cAMP level in the forskolin-treated flukes remained elevated throughout the 45-min incubation period. This is in contrast to the previously described effect of 5-HT incubation on cAMP levels which after rising sharply quickly decreased back towards control values [2]. The increased cAMP levels in forskolin-treated flukes was concentration dependent. Table 2 shows that incubation with 300 μ M forskolin gave the maximum increase in cAMP. This concentration dependence was similar to that found for forskolin activation of adenylate cyclase in fluke particles (Fig.

Table 1. Effect of forskolin on activation of fluke adenylate cyclase by Gpp(NH)p and GTP γ S*

Assay conditions	Adenylate cyclase activity (pmoles/mg·min)	Expected activity if additive
No additions (basal conditions)	6.5	
Forskolin	120	
1 μ M Gpp(NH)p	45	
1 μ M Gpp(NH)p + forskolin	170	165
10 μ M Gpp(NH)p	110	
10 μ M Gpp(NH)p + forskolin	220	230
100 μ M Gpp(NH)p	130	
100 μ M Gpp(NH)p + forskolin	220	250
100 nM GTP γ S	450	
100 nM GTP γ S + forskolin	360	570
1 μ M GTP γ S	1000	
1 μ M GTP γ S + forskolin	720	1120
10 μ M GTP γ S	1100	
10 μ M GTP γ S + forskolin	890	1220

* Fluke particles were assayed for adenylate cyclase activity as described in Methods and Materials under the assay conditions indicated. The concentration of forskolin used was 100 μ M. The values given are means from duplicate samples of a representative experiment.

Table 2. Effect of forskolin on cAMP levels in intact flukes

Forskolin (M)	[cAMP] in intact flukes (pmoles/mg protein)	
	Expt. 1	Expt. 2
None	43.9	26.4
10^{-5}	66.7	26.4
10^{-4}	69.7	37.1
3×10^{-4}	128.6	43.5
10^{-3}	106.0	40.0

* Liver flukes were incubated at 37° in media containing the indicated concentrations of forskolin. A stock solution of 0.01 M forskolin in 95% ethanol was used to prepare the indicated concentrations. Thus, the final concentration of forskolin in media contained a carryover amount of ethanol (e.g. 0.095% in the case of 10^{-5} M forskolin). After incubation for 5 min in Expt. 1 or 15 min in Expt. 2, the cAMP content of the flukes was determined by radioimmunoassay as described in Methods and Materials. The results given are mean values from duplicate samples.

1). Also similar was the finding that LSD and forskolin acted together synergistically to increase cAMP levels in the intact flukes (Table 3, Expt. 2). These findings suggest that the increases in cAMP levels due to forskolin are the direct result of forskolin's activation of adenylate cyclase.

cAMP and fluke motility. During the course of these studies, it was found that the incubation of intact flukes with forskolin at concentrations above 50 μ M markedly stimulated their rhythmical movement. Previous reports [2] from our laboratory suggested a correlation between increased cAMP levels caused by serotonin and increased fluke motility. This is consistent with the present finding shown in Table 3 indicating that increased fluke motility occurred at forskolin concentrations where increased cAMP levels were found. Furthermore, it can be seen in Table 3 that incubation of flukes with 5-HT or with 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, also increased both cAMP levels

and fluke motility [13]. An increase in cAMP levels was not observed after incubation with LSD at concentrations which markedly enhanced fluke motility. However, in the presence of 3-isobutyl-1-methylxanthine or forskolin, fluke cAMP levels were increased by the addition of LSD.

DISCUSSION

The liver fluke *Fasciola hepatica* contains a very active adenylate cyclase that is controlled by at least two membranous components, serotonin receptors and GTP-proteins [1-3, 14]. Enzyme activity is activated by serotonin and its agonists through the receptors, and by the hydrolysis-resistant GTP analogues through the GTP-protein. The diterpene, forskolin, was shown to activate adenylate cyclases from many sources and to potentiate receptor-mediated activation of the enzyme [15]. The results presented here clearly show that forskolin activated adenylate cyclase in membrane particles from the liver fluke, and that it raised the level of cyclic AMP in intact organisms. Neither effect appears to be receptor-mediated since activation was not blocked by bromo-LSD, a potent antagonist of the serotonin receptors in this system [2, 3]. Furthermore, forskolin showed no effect on the binding of [3 H]LSD to the serotonin receptors in the fluke particles. The diterpene, however, was shown to potentiate receptor-mediated activation of the enzyme by serotonin or by LSD. Forskolin increased maximal serotonin response and lowered the apparent K_A for activation by the indoleamine. Forskolin, therefore does not seem to require the serotonin receptors for its action but could modulate the regulation of the cyclase by the indoleamine. The effect on the fluke cyclase system here appears to be similar to what was reported on receptor-activated adenylate cyclase from mammalian cells. The exact locus of forskolin action in all these enzyme systems remains to be elucidated. The discovery by Seamon and Daly [16] that a normal GTP-protein is not required for forskolin activation of adenylate cyclase in the *cyc*⁻ mutant of S49 mouse

Table 3. Effects of forskolin and other agents on cAMP levels and motility in intact flukes*

Expt.	Incubation conditions	[cAMP] (pmoles/mg protein)	Motility
1	Control	34	1+
	1 mM 5-HT	650	4+
	100 μ M Forskolin	91	3+
2	Control	45	1+
	100 μ M Forskolin	68	3+
	1 μ M LSD	47	4+
	1 μ M LSD + 100 μ M forskolin	93	4+
3	Control	41	1+
	2 mM IBMX	64	3+
	2 mM IBMX + 1 μ M LSD	76	4+

* Liver flukes were incubated at 37° for 5 min under the specified conditions. Following incubation, the flukes were analyzed for motility using a subjective scale ranging from 1+ (little or no movement) to 4+ (very marked undulations and constant hyperactive movement) as we described previously [2, 13]. The cAMP content of the flukes was determined by radioimmunoassay as described in Methods and Materials. The results given are the means of duplicate samples.

lymphoma while guanine nucleotides modify forskolin-activated cyclase from the same cells [17] introduced uncertainty about the site of action of the diterpene. More recently, Gpp(NH)p was shown to inhibit the forskolin-activated cyclase in rat adipocyte membranes [18], giving the nucleotide an inhibitory effect as well as an activating one. In the fluke cyclase system, stimulation by Gpp(NH)p and by forskolin was almost additive. The inhibitory component for Gpp(NH)p on forskolin activation appears to be missing in the fluke enzyme system. However, this conclusion must be considered tentative in view of the finding that activation of adenylate cyclase by forskolin and GTP γ S was less than activation by GTP γ S alone. This finding, when added to the results reported in Fig. 6 that at high fluoride concentration forskolin decreased activation of the enzyme, suggests that the effect of forskolin may be due, at least in part, to its ability to modify the interaction between the regulatory and the catalytic components of the membranous enzyme complex. Katada *et al.* [19] reported that both NaF and GTP γ S are less effective activators in the presence of high concentrations of forskolin, using human platelet membranes.

The ability of forskolin to elevate fluke cAMP levels and increase fluke motility provides new evidence that cAMP may have a role in the regulation of fluke motility. Previous studies [2, 5] indicated that 5-HT, LSD, and other agents which activated adenylate cyclase also increased fluke motility. Furthermore, 2-bromo-LSD inhibited 5-HT-stimulated adenylate cyclase and paralyzed the flukes. Since all these agents acted through the 5-HT receptor, it could have been argued that the effect on motility was a receptor function that was not mediated by cAMP. The ability of forskolin to elevate fluke cAMP levels and increase fluke motility provides a new example of a correlation between the two effects. The significance of the effect of forskolin in this respect is that it appears to increase cAMP levels by a non-serotonin receptor mediated mechanism.

The dual effect of forskolin on motility and cAMP levels in the flukes may further implicate the cyclic nucleotide in the neuromuscular function of these parasites.

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