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Forskolin Does Not Activate Sperm Adenylate Cyclase

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SUMMARY

Forskolin, a potent activator of adenylate cyclase, has been proposed to activate this enzyme by a direct interaction with the catalytic subunit. To test this hypothesis, we examined the effects of forskolin on sperm cyclic AMP content and sperm adenylate cyclase activity. Forskolin or cholera toxin did not increase cyclic AMP content in either bull or boar sperm, whereas the inhibitors of phosphodiesterase, caffeine and methylisobutylxanthine, significantly increased sperm cyclic AMP content. Forskolin, NaF, and guanylimidodiphosphate did not activate the adenylate cyclase of either sperm membranes or cytosol. When homogenates of rat, guinea pig, or bull testes were centrifuged at 100,000 × *g*, the supernatant was found to contain a forskolin-stimulated adenylate cyclase. Further centrifugation of this 100,000 × *g* supernatant fraction at 250,000 × *g* for 3 hr quantitatively sedimented the forskolin-sensitive enzyme activity. We conclude that forskolin does not activate either the cytosolic or membrane-bound adenylate cyclase of mammalian sperm.

INTRODUCTION

Forskolin was isolated from the Indian plant, *Coleus Forskohlii*, and characterized by Bhat *et al.* (1). This diterpene has several pharmacological effects when examined *in vivo* and *in vitro*. It has a potent positive inotropic action, it lowers the blood pressure in dogs and cats, and it exerts an antihypertensive action in rats (2, 3). Forskolin exerts a direct relaxant effect on smooth muscle preparations *in vitro* (3). A subsequent study of both naturally occurring derivatives of forskolin and several semisynthetic derivatives demonstrated that the optimal structural requirements for pharmacological activity appeared to be found in the structure of forskolin (4). No antagonists of forskolin have been reported to date (4).

Following the demonstration of the cardiovascular activity of forskolin (2, 3), Metzger and Lindner (5) reported that the forskolin-mediated increase in contractile force in isolated atria of guinea pigs is correlated with increased activity of the cyclic AMP-dependent protein kinase as measured by the protein kinase activity ratio. They extended this observation by showing that forskolin activates adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] of rat heart and liver. Subsequently, forskolin has been shown to be a potent activator of adenylate cyclase from a variety of mammalian tissues (6). Unlike cholera toxin, fluoride, and guanyl nucleotides, activation of adenylate cyclase by forskolin appears to be fully reversible (7, 8). In addition, it has been reported that forskolin-mediated activation of adenylate cyclase does not require the presence of a functional guanine nucleotide regulatory protein (*N* protein), suggesting that the diterpene may interact directly with the catalytic subunit of adenylate cyclase (9). Other studies from the same laboratory (8) and by others (10), however, suggest that full expression of the effects of forskolin may require the *N* protein.

To determine whether forskolin activates adenylate cyclase by a direct interaction with the catalytic subunit, we have studied the effects of forskolin on the cyclic AMP content and on adenylate cyclase activity of mammalian sperm. Adenylate cyclase of sperm is not activated by hormones, fluoride, guanyl nucleotides (11-13), or cholera toxin (14); thus the enzyme represents a model of a "bare" catalytic subunit. A preliminary report demonstrating that forskolin does not increase the cyclic AMP content of viable sperm nor activate the sperm adenylate cyclase in broken-cell preparations has been published (15).

METHODS

Ejaculated semen was collected from bulls and boars that were maintained by the Department of Animal Science, University of Missouri. The semen was diluted with Krebs-Ringer-phosphate medium (pH 7.4) (16) at a ratio of one part semen to two parts medium. Sperm were sedimented by centrifugation at 1,000 × *g* for 5 min, followed by

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resuspension in medium. The sperm were washed by three cycles of centrifugation-resuspension in Krebs-Ringer-phosphate medium.

Effects of drugs on motility and cyclic AMP content of sperm were measured by preincubating sperm at 37° in Krebs-Ringer-phosphate medium for 15 min, followed by incubation with forskolin, caffeine, MIX,[4] or cholera toxin for the times specified in the respective experiments. Motility was estimated using the procedure of Garbers *et al.* (17). After incubation of sperm with the above drugs, 0.5-ml samples of sperm plus medium were transferred to 0.5 ml of 100 mM sodium acetate (pH 4.0) and maintained at 100° for 4 min. This mixture was centrifuged and the supernatant was used to measure cyclic AMP by radioimmunoassay (18, 19).

Plasma membrane preparations of washed sperm were isolated by layering 10 ml of resuspended sperm over 50 ml of 1 M sucrose/0.15 M NaCl and centrifuging at 2,200 × *g* for 30 min (20). The pelleted sperm were resuspended in 2 ml of 0.15 M NaCl/5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) and homogenized with a Teflon pestle/glass tube. The homogenate was then fractionated by layering 10 ml of homogenate over a gradient consisting of 14 ml of 0.25 M sucrose/0.15 M NaCl and 14 ml of 1.5 M sucrose/0.15 M NaCl and centrifuging at 110,000 × *g* for 2 hr in a Beckman SW 27 rotor. The membrane fraction was collected at the 0.25 M–1.5 M sucrose interface while the cytosol (soluble) fraction was removed from the top of the gradient. Samples were stored at −70° until assayed for adenylate cyclase activity.

Subcellular fractions of testes (rat, guinea pig, and bovine) were prepared according to the technique reported by Neer (21). The tissue was homogenized (1 g of tissue per 4 ml of medium) in a medium containing 0.15 M NaCl, 75 mM sucrose, and 5 mM Tris-HCl (pH 7.4) using a Teflon pestle/glass tube. A sample of homogenate was removed and stored at −70° for measurement of protein and adenylate cyclase activity. The remaining homogenate was centrifuged at 100,000 × *g* for 1 hr and the supernatant was removed. The pellet was then resuspended in medium and centrifuged at 100,000 × *g* for 1 hr. This procedure was repeated for a total of three washes. The supernatants from the 100,000 × *g* centrifugation were combined, and the pellet was resuspended in medium and stored at −70°. The supernatant at this step is the "water-soluble" adenylate cyclase preparation used by Seamon and Daly (9) according to the procedure of Neer (21). An aliquot of the 100,000 × *g* supernatant was stored at −70° for assay of adenylate cyclase activity, and the remaining supernatant fraction was then centrifuged at 250,000 × *g* for 3 hr. This centrifugation yielded a clear supernatant fraction and a pellet. The pellet was resuspended in medium, and both fractions were stored at −70° until assayed for adenylate cyclase activity.

These preparations from sperm and testes were assayed for adenylate cyclase activity as described previously for other tissues (22). Briefly stated, the reaction mixture (75 μl) contained (in millimolar concentration) 50 Tris-HCl (pH 7.5), 6.7 $MgCl_2$, 12 creatine phosphate, 0.1 cyclic AMP, 16 caffeine, and 1.2 [α-^{32}P]ATP (5 × 10^{12} cpm/mole), plus bovine serum albumin (266 μg/ml), creatine phosphokinase (13.3 units/ml), and 50–100 μg of protein. Incubation was carried out at 30° for either 15 or 20 min. Under these experimental conditions, the formation of product was linear. Cyclic [^{32}P]AMP was separated from other ^{32}P-labeled nucleotides by the method of Salomon *et al.* (23). Recovery of cyclic AMP, monitored with [^{3}H]cyclic AMP, was 60–70%. Protein was measured by the procedure of Lowry *et al.* (24). Data are presented as the means ± standard error of the mean of replicate assays and are representative experiments. Statistical analysis of the data was performed using Student's *t*-test (25).

Forskolin was obtained from Calbiochem-Behring Corporation (LaJolla, Calif.). Caffeine, MIX, and cholera toxin were obtained from Sigma Chemical Company (St. Louis, Mo.). [α-^{32}P]ATP (10–30 Ci/mmole) was obtained from ICN Pharmaceuticals (Irvine, Calif.), and [^{3}H]cyclic AMP (38 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Other materials were obtained from various suppliers and were the highest grade available.

[4] The abbreviations used are: MIX, methylisobutylxanthine; Gpp(NH)p, guanylimidodiphosphate.

RESULTS

To determine whether forskolin activates adenylate cyclase of sperm, we measured the effects of forskolin on cyclic AMP levels in intact, viable sperm from bulls and boars. We found that 100 μM forskolin had no effect on the cyclic AMP content of either bovine (Fig. 1) or porcine (Fig. 2) sperm. Cholera toxin also was ineffective, whereas the inhibitors of cyclic AMP phosphodiesterase, caffeine, and MIX significantly increased the cyclic AMP content of sperm from both species. Forskolin in the presence of either caffeine or MIX also did not increase cyclic AMP levels over that observed with either caffeine or MIX alone (data not shown). Moreover, a qualitative increase in cell motility was obvious after treatment with caffeine or MIX, but not with forskolin.

Direct measurement of sperm adenylate cyclase activity was carried out by using homogenates of sperm. Data from these experiments are summarized in Figs. 3 (bovine) and 4 (porcine). Forskolin (100 μM), Gpp(NH)p (10 μM), and NaF (10 mM) did not increase adenylate cyclase activity in either bovine or porcine sperm homogenates. The diterpene was also ineffective at 10 and 300 μM concentrations (data not shown), whereas 15 mM manganese markedly stimulated enzyme activity. Forskolin at 100 μM in the presence of Mn^{2+} did not further increase the adenylate cyclase activity over that observed with Mn^{2+} alone (data not shown). Porcine sperm homogenates were fractionated by differential centrifugation to yield both membrane (particulate) and soluble forms of

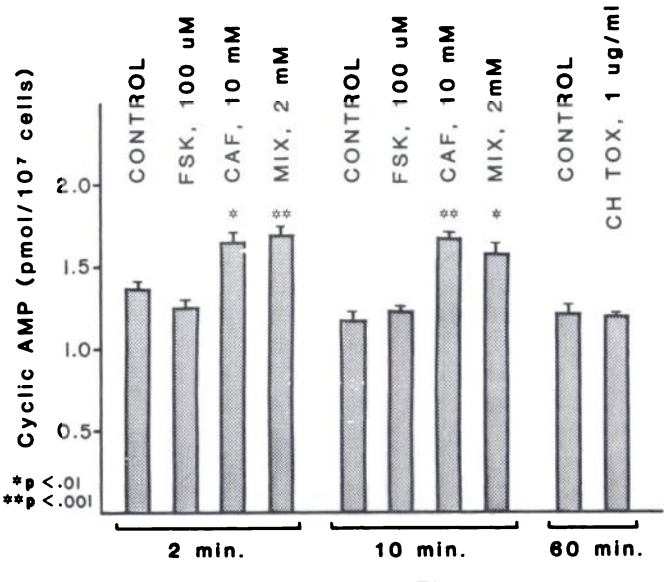

FIG. 1. *Effects of forskolin, phosphodiesterase inhibitors, and cholera toxin on bovine sperm cyclic AMP content*

Sperm suspended in Krebs-Ringer-phosphate buffer (pH 7.4) at a density of 10^7 cells/ml were incubated at 37° for 10 min. Vehicle or drugs were then added and the incubation was continued for the indicated times. Cyclic AMP content is cells plus medium, and the data are expressed as the means ± standard error of the mean of four replicate samples; *p* values are relative to control. *FSK*, forskolin; *CAF*, caffeine; *CH TOX*, cholera toxin.

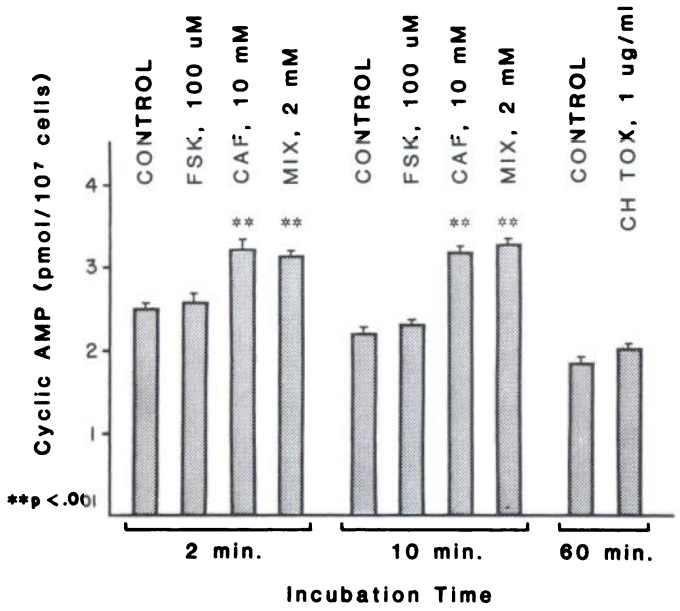

FIG. 2. *Effects of forskolin, phosphodiesterase inhibitors, and cholera toxin on porcine sperm cyclic AMP content*
The conditions for this experiment are identical with those described in the legend to Fig. 1; $n = 8$.

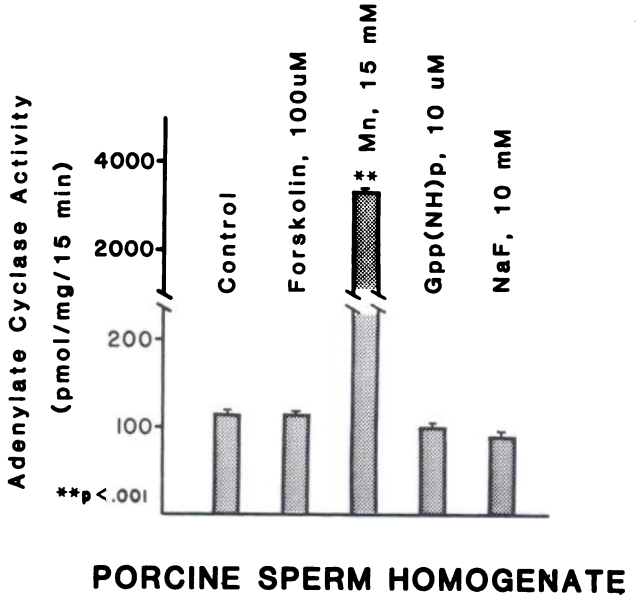

FIG. 4. *Adenylate cyclase activity of porcine sperm homogenates*
The conditions are the same as those described in the legend to Fig. 3.

adenylate cyclase. Forskolin, Gpp(NH)p, and NaF did not activate the adenylate cyclase of either subcellular fraction, whereas Mn^{2+} was effective in activating both fractions of the sperm enzyme (Fig. 5).

These data, which indicate that forskolin is ineffective in activating adenylate cyclase of mammalian sperm, contradict the observation of Seamon and Daly (9), who reported that forskolin activated a "water-soluble" adenylate cyclase prepared from rat testes. This "soluble" enzyme preparation is thought to be derived from sperm (12). To clarify this apparent discrepancy, we carried out a series of experiments designed to study the effects of forskolin, Gpp(NH)p, NaF, and Mn^{2+} on the adenylate cyclase of rat, guinea pig, and bovine testes. In most experiments we measured the adenylate cyclase activity of the tissue homogenate as well as the "water-soluble" (100,000 × *g* supernatant) preparation used by Seamon

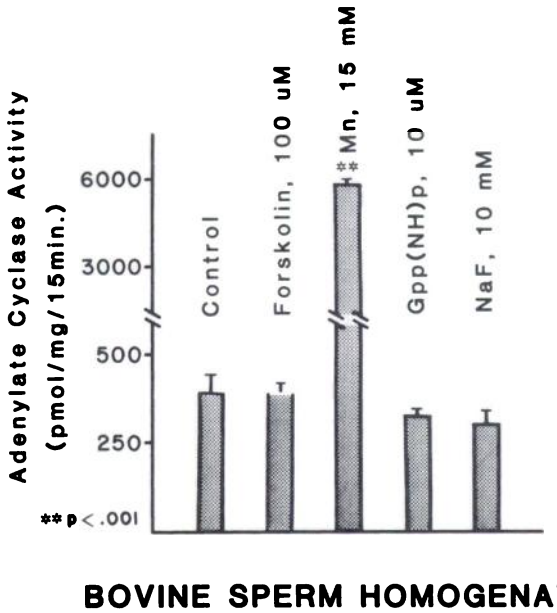

FIG. 3. *Adenylate cyclase activity of bovine sperm homogenates*
The preparation of homogenates from ejaculated, washed sperm is described under Methods. The data are expressed as the means ± standard error of the mean of four replicate assays of one homogenate. This experiment was repeated with other sperm homogenates to confirm these data; *p* values are relative to control.

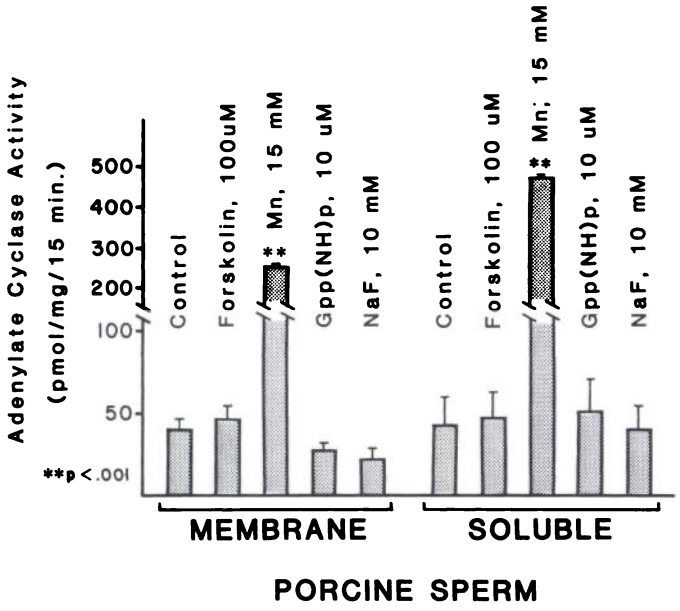

FIG. 5. *Adenylate cyclase activity of membrane-bound and soluble fractions from porcine sperm*
The technique for isolation of membrane and cytosol portions from ejaculated, washed sperm is described under Methods. The data are the means ± standard error of the mean of four replicate assays from one representative experiment which has been repeated and confirmed; *p* values are relative to control.

and Daly (9). In addition, the "water-soluble" fraction was subjected to a second centrifugation at 250,000 × *g* for 3 hr, which yielded a clear supernatant and a particulate fraction (see Methods).

Homogenates of rat and guinea pig testes exhibited adenylate cyclase activity that was stimulated significantly by forskolin, Mn^{2+}, Gpp(NH)p, or NaF (Table 1). When testicular homogenates were centrifuged at 100,000 × *g*, we obtained particulate fractions which had adenylate cyclase activity that was stimulated by forskolin, Mn^{2+}, Gpp(NH)p, and NaF. The "water-soluble" adenylate cyclase (100,000 × *g* supernatant) of rat testes was not activated by forskolin, Gpp(NH)p, or NaF but was markedly activated by Mn^{2+}. However, forskolin significantly increased the adenylate cyclase activity of 100,000 × *g* supernatants from both guinea pig and bovine testes. The adenylate cyclase in this subcellular fraction of guinea pig testes was activated much less by Gpp(NH)p and NaF whereas these activators were ineffective in the corresponding bovine testes fraction. When the 100,000 × *g* supernatants from all three species were centrifuged at 250,000 × *g*, the resulting supernatants contained a truly water-soluble adenylate cyclase that was not activated by forskolin, Gpp(NH)p, or NaF. Moreover, the particulate fractions from those centrifugations were found to contain forskolin-stimulated adenylate cyclase activity. It is of interest that, although forskolin significantly stimulated the adenylate cyclase in the 250,000 × *g* pellet from all three species, there was no significant stimulation of enzyme activity observed with either Gpp(NH)p or NaF. All of these subcellular fractions contained Mn^{2+}-stimulated adenylate cyclase activity. These data suggested that the putative "water-soluble" adenylate cyclase derived from rat testes according to the techniques used by Seamon and Daly (9) probably contained a forskolin-stimulated adenylate cyclase that could be sedimented by centrifugation at 250,000 × *g* for 3 hr. Therefore, the discrepancy between the data of Seamon and Daly (9) and those reported here are apparently reconciled. Clearly, the "bare" catalytic unit represented by either the soluble or the membrane-bound adenylate cyclase of sperm is not activated by forskolin.

DISCUSSION

The results of this study clearly document that forskolin does not increase cyclic AMP levels in intact, viable mammalian sperm, nor does it increase adenylate cyclase activity in subcellular fractions of sperm. It appears that the adenylate cyclase of sperm, whether membrane-bound or soluble, is a catalytic subunit that is funtionally devoid of the *N* subunit and hormone receptors, since the enzyme is not regulated by hormones, fluoride, guanyl nucleotides, or cholera toxin (11–14). Moreover, the sperm enzyme is greatly activated by Mn^{2+}, similar to other adenylate cyclase preparations which lack a functional *N* subunit (26–28). Forskolin did not activate sperm adenylate cyclase in the presence of either Mg^{2+} or Mn^{2+}, which confirms the observation made by Stengel *et al.* (29). The failure of forskolin to activate the adenylate cyclase of sperm suggests but certainly does not prove that forskolin activates the adenylate cyclase of other eukaryotic cells by interacting with a component of the adenylate cyclase system other than the catalytic subunit.

Our data also provide an explanation for the observation by Seamon and Daly (9), who reported that forskolin activated the adenylate cyclase in a putative "water-soluble" (100,000 × *g* supernatant) fraction from rat testes. It is evident that preparations of this type,

TABLE 1

Effect of forskolin on testicular adenylate cyclase (see Methods)

Species and addition	Adenylate cyclase activity				
	Hemogenate	100,000 × *g* supernatant	100,000 × *g* pellet	250,000 × *g* supernatant	250,000 × *g* pellet
			pmoles/mg/20 min		
Rat					
None	170 ± 36	70 ± 9	34 ± 7	64 ± 2	88 ± 8
Forskolin, 100 μM	931 ± 242[a]	71 ± 10	319 ± 82[a]	53 ± 3	119 ± 6[a]
$MnCl_2$, 15 mM	1849 ± 321[b]	2477 ± 310[b]	257 ± 73[a]	2475 ± 455[b]	2536 ± 390[a]
Gpp(NH)p, 10 μM	292 ± 42[a]	69 ± 10	84 ± 20[a]	63 ± 2	90 ± 15
NaF, 10 mM	693 ± 181[a]	65 ± 8	709 ± 161[a]	61 ± 5	110 ± 22
Guinea pig					
None	343 ± 6	7 ± 1	769 ± 26	10 ± 3	47 ± 2
Forskolin, 100 μM	1283 ± 31[c]	52 ± 1[c]	2716 ± 81[c]	12 ± 2	150 ± 7[c]
$MnCl_2$, 15 mM	904 ± 13[c]	629 ± 8[c]	1311 ± 28[c]	229 ± 8[c]	3699 ± 106[c]
Gpp(NH)p, 10 μM	547 ± 20[c]	12 ± 1[b]	1316 ± 25[c]	10 ± 2	45 ± 2
NaF, 10 mM	664 ± 24[c]	18 ± 2[b]	1466 ± 34[c]	18 ± 2	44 ± 2
Bovine					
None	Not assayed	13 ± 1	Not assayed	9 ± 1	63 ± 1
Forskolin, 100 μM		44 ± 1[c]		12 ± 1	150 ± 7[c]
$MnCl_2$, 15 mM		364 ± 10[c]		169 ± 4[c]	2219 ± 57[c]
Gpp(NH)p, 10 μM		14 ± 1		6 ± 2	74 ± 6
NaF, 10 mM		14 ± 1		13 ± 1	77 ± 2

[a] $p < 0.05$ versus basal.
[b] $p < 0.01$ versus basal.
[c] $p < 0.001$ versus basal.

whether from rat, guinea pig, or bull sperm, contain a small amount of particulate adenylate cyclase that is activated by forskolin. This forskolin-dependent enzyme activity sedimented when the 100,000 × *g* supernatant fraction was centrifuged at 250,000 × *g* for 3 hr. The 250,000 × *g* supernatant fraction contained a truly water-soluble adenylate cyclase catalytic subunit that was not activated by forskolin. This conclusion is supported by recent experiments which showed a lack of forskolin stimulation of the soluble cyclase prepared from rat or ram testicular homogenates by gel filtration (29, 30).

Seamon and Daly (9) proposed that the activation of adenylate cyclase by forskolin does not require the presence of a functional *N* subunit since forskolin activates the adenylate cyclase of a mutant cell line of the S49 mouse lymphoma (S49 *cyc*$^-$) which lacks a functional *N* subunit. However, more recent work using the *cyc*$^-$, H21a, and *unc* S49 mutants indicates that the *N* subunit has some functional role in the expression of the full activity of forskolin (10). The well-characterized property of forskolin at low concentrations to potentiate or augment the cyclic AMP response of intact cells or tissue to hormonal stimulation also implicates the *N* subunit as a possible site of action of forskolin (8, 10).

Ross (28) reported that forskolin activated the adenylate cyclase of a detergent-solubilized adenylate cyclase catalytic subunit isolated from rabbit liver. This preparation had been chromatographically separated from a functional *N* subunit, and the resolved catalytic subunit was not activated by fluoride or guanyl nucleotide (GTPγS). Recently Pfeuffer and Metzger (31) prepared a forskolin-Sepharose affinity gel, which was used to isolate a catalytic subunit that was free of functional *N* subunit activity. They further demonstrated that forskolin activated the catalytic subunit when *N* protein was not present. Data from both of these studies (28, 31) suggest that forskolin can activate adenylate cyclase when the *N* subunit is absent. Thus, it appears possible that forskolin may not directly interact with either the catalytic or the *N* subunit, but instead interacts with another macromolecule ("receptor") that influences catalytic activity. It is possible that this putative regulatory factor is present in the "resolved" catalytic subunit fraction studied by either Ross (28) or Pfeuffer and Metzger (31) but is absent or functionally inactive in sperm.

An alternate hypothesis is that the sperm catalytic subunit may represent an improper model for the eukaryotic adenylate cyclase. The low molecular weight soluble enzyme may represent only a fragment of the catalytic subunit derived from degradation of the membrane-bound enzyme by proteolysis. This may not be the case for the membrane-bound enzyme, since the adenylate cyclase of ram sperm has been shown to interact with the *N* subunit of human erythrocyte ghosts in a manner that appeared to produce a functional coupling between erythrocyte *N* protein and the sperm catalytic subunit (32). After reconstitution of ram sperm membranes with erythrocyte ghost membranes, the adenylate cyclase was activated by guanyl nucleotides, fluoride, and the hormonal agonist, prostaglandin E_1. Stengel *et al.* (29) recently reported that when human erythrocyte ghost membranes containing functional *N* subunit are incubated with ram sperm membranes containing the catalytic subunit, the sperm enzyme is subsequently activated by forskolin. It remains a viable possibility that reconstitution of erythrocyte membranes with sperm membrane adenylate cyclase may produce a forskolin-stimulated catalytic enzyme activity which may be unrelated to the putative reconstituted *N* protein-catalytic subunit interaction. In this case it may be hypothesized that the appearance of a forskolin-stimulated sperm adenylate cyclase is due to reconstitution of a "forskolin-receptor" macromolecule with the sperm catalytic subunit in a functional manner, as proposed by Stengel *et al.* (29). It is possible that our studies may have provided an example of this type. When the 100,000 × *g* supernatant was centrifuged at 250,000 × *g* we found that the pellet fraction was activated by forskolin and by Mn^{2+} but was not stimulated by either Gpp(NH)p or NaF. This fraction may represent an example of the catalytic subunit that is functionally associated with a "forskolin-receptor" but devoid of the *N* subunit. This type of mechanism should be considered in the interpretation of the studies to date on the molecular site of forskolin activation of adenylate cyclase (9, 10, 29, 31, 32) and in the design of future experiments to explore the molecular site of action of forskolin.

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