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Stimulation of Adenylate Cyclase by Water-Soluble Analogues of Forskolin

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SUMMARY

Analogues of forskolin that are more soluble in water than forskolin have been synthesized and tested for their ability to interact with adenylate cyclase. These analogues are esterified with various heterocyclic amino acids at the 6β-hydroxyl position of forskolin or at the 6β-hydroxyl or 7β-hydroxyl position of 7-desacetyl forskolin. Analogues were tested for their ability to activate rat brain adenylate cyclase, activate detergent-solubilized rat brain adenylate cyclase, increase cyclic AMP in intact S49 wild-type cells, and inhibit the binding of ^{3}H-forskolin to rat brain membranes. Forskolin activated rat brain adenylate cyclase with an EC_{50} of 4 μM and increased cyclic AMP in intact S49 cells with an EC_{50} of 5 μM. Analogues esterified at the 7β-hydroxyl position had EC_{50} values that ranged from 4 μM to 15 μM for activating adenylate cyclase in membranes and solubilized preparations, and for increasing cyclic AMP in S49 cells. Analogues esterified at the 6β-hydroxyl position with no acyl group at the 7β-hydroxyl position were generally less potent than the corresponding 7-acyl analogues with EC_{50} values that ranged from 30 μM to 100 μM. Interestingly, the diacyl analogues of forskolin containing an acetate group at the 7β-hydroxyl position and esterified with heterocyclic amino acids at the 6β-hydroxyl position were very potent at stimulating adenylate cyclase, with EC_{50} values that ranged from 1 μM to 25 μM. The 7-acyl analogues and the 6,7-diacyl analogues inhibited the binding of ^{3}H-forskolin to rat brain membranes with IC_{50} values that ranged from 20 μM to 70 μM, while the 6-acyl analogues had much higher IC_{50} values that ranged from 100 nM to 375 nM. Aqueous solutions of forskolin were also produced by dissolving forskolin in solutions of hydroxypropyl-γ-cyclodextrin. These aqueous solutions of forskolin were equipotent with alcoholic solutions of forskolin in stimulating adenylate cyclase. In conclusion, water-soluble derivatives of forskolin may be useful for increasing cyclic AMP in broken cell preparations or in intact cell preparations where the presence of organic solvents, which are necessary to solubilize forskolin, are detrimental. Alternatively, aqueous solutions of forskolin can be produced by dissolving forskolin in solutions of hydroxypropyl-γ-cyclodextrin.

The diterpene forskolin was first discovered due to its ability to produce cardiotonic effects (1). it was quickly recognized that forskolin's pharmacological effects, which included relaxation of smooth muscle and positive inotropic effects, resulted from its ability to activate adenylate cyclase (2–4). Forskolin has since been used extensively in studies relating to the role of adenylate cyclase and cyclic AMP in regulating cellular functions and physiology (5, 6). Forskolin activates almost all mammalian adenylate cyclases in intact tissues and cells, in membrane preparations, and in solubilized preparations. The qualitative characteristics for the activation of adenylate cyclase by forskolin in different preparations are similar, with forskolin exerting its effects with a potency in the μM range. High affinity interactions of forskolin with adenylate cyclase have also been described. Forskolin and hormones that stimulate adenylate cyclase can produce synergistic stimulations of adenylate cyclase at concentrations of forskolin that are considerably lower than those required for the direct activation of adenylate cyclase in the absence of hormone (7–9). Binding sites for forskolin have also been described in rat brain membranes and human platelet membranes which have a K_d for forskolin of 20 nM (10–13). It has been suggested that these sites may be associated with a ternary complex of the catalytic subunit of adenylate cyclase and the stimulatory regulatory protein which is responsible for mediating hormonal stimulation of adenylate cyclase (11, 12).

Effects of forskolin have been described that are not compatible with a simple model where forskolin only stimulates adenylate cyclase. Low concentrations of forskolin have been shown in some cases to be inhibitory to adenylate cyclase (14–16). Forskolin has also been shown to inhibit glucose transport in erythrocytes and adipocytes, and it has been suggested that this inhibition is due to a direct inhibitory effect of forskolin on the glucose transporter (17, 18). Forskolin inhibits the

ABBREVIATIONS: MOPS, 4-morpholineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

nicotinic receptor-mediated influx of Rb^{2+} in rat pheochromocytoma cells (18). It was suggested that the inhibition by forskolin was due to a disruption of membrane structure since the inactive analogue of forskolin, 1,9-dideoxyforskolin, also inhibited the receptor-mediated influx of Rb^{2+}.

Forskolin is a very lipophilic compound with a limited water solubility. The limited solubility of forskolin in water has made it difficult to analyze forskolin activation curves. In many cases, effects due to forskolin do not exhibit saturation with respect to forskolin concentrations. Part of this difficulty may be due to the limited solubility of forskolin. However, it has also been shown that organic solvents used to solubilize forskolin can also inhibit forskolin's activation of adenylate cyclase (20, 21). Water-soluble derivatives of forskolin would therefore be of immense value in studies relating to cyclic AMP and the physiological role of cyclic AMP. A number of water-soluble derivatives of forskolin have been synthesized, and, in this paper, we have tested them for their ability to increase cyclic AMP in intact cells and also to stimulate membrane-associated and detergent-solubilized adenylate cyclase. A procedure for solubilizing forskolin in aqueous solutions of hydroxypropyl-γ-cyclodextrin is also described and shown to be effective for making solutions of forskolin in the absence of organic solvents.

Experimental Procedures

Materials. Forskolin was obtained from Calbiochem; ATP, creatine phosphokinase, phosphocreatine, and cyclic AMP were purchased from Sigma; the S49 wild-type cells were the kind gift of Dr. Susan Beckner, National Cancer Institute, National Institutes of Health, Frederick, MD; [2-^{3}H] adenine (17 Ci/mmol), [α-32P]ATP, cyclic [^{3}H]AMP (31.2 Ci/mmol), and [^{3}H]forskolin (24 Ci/mmol) were obtained from New England Nuclear, Boston, MA; and hydroxypropyl-γ-cyclodextrin was the generous gift of Dr. Josef Pitha, National Institutes of Health, NIA/GRC, Baltimore, MD. The water-soluble forskolin analogues referred to in this paper are described in Indian Patent Applications Nos. 1221B011/85 dated May 3, 1985 and 1331B011/86 dated April 29, 1986; and German Applications Nos. P.3535086.5 dated October 2, 1985 and HOE 861F153. Compound **3** is now available from Calbiochem. All other reagents were from standard sources.

Membrane preparation. Membranes were prepared from rat forebrain as previously described (22). One rat forebrain was homogenized in 10 ml of 0.32 M sucrose. The homogenate was centrifuged at 1,000 × *g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 10,000 × *g* for 10 min, resuspended in 10 ml of 50 mM Tris-HCl (pH 7.5), and homogenized in a Dounce homogenizer.

Membrane solubilization. Membranes were resuspended in 5 ml of a solubilization buffer containing 10 mM MOPS, pH 7.4, 5 mM, Lubrol, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol. The membranes were homogenized with a Dounce homogenizer and the suspension was stirred on ice for 1 hr. The membrane suspension was centrifuged at 30,000 × *g* for 30 min and the pellet was discarded. Proteins were determined by the method of Lowry *et al.* (23).

Adenylate cyclase activity. Adenylate cyclase activity was determined as previously described (22). Crude membranes or detergent-solubilized proteins (25 μl, 100 μg of protein/assay) were incubated in 0.25 ml of a solution containing 50 mM Tris HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM dithiothreitol, 2 units of creatine phosphokinase, 2 mM creatine phosphate, and 0.1 mM ATP containing 1 μCi of [α-32p]ATP. Solubilized adenylate cyclase was assayed in the presence of 5 mM $MnCl_2$. Incubations were for 10 min at 30° and were terminated with 0.5 ml of 10% trichloroacetic acid and 0.25 ml of a solution containing about 10,000 cpm of cyclic [^{3}H]AMP. Cyclic AMP was determined using the method of Salomon *et al.* (24).

Measurement of cyclic AMP in S49 wild-type cells. The S49 wild-type cells were grown at 37° with Dulbecco's minimal essential medium which had been supplemented with glutamine, 10% fetal calf serum, and antibiotics. The cell density was maintained at 0.7–1.2 × 10^6 cells/ml. Cells were washed and resuspended in serum-free medium containing 10 mM HEPES, pH 7.5, at a density of 20 × 10^6 cells/ml. The cell suspension was incubated for 60 min at 37° in medium containing 10 μCi of [2-^{3}H]adenine/ml. Cells were washed three times with fresh medium and resuspended at the same cell density in the same medium containing 1 mM 3-isobutyl-1-methylxanthine. The cells were incubated with the indicated agents for 30 min at 24° in a final volume of 150 μl. Incubations were stopped by the addition of 0.5 ml of 10% trichloroacetic acid and 0.25 ml of a 1.5 mM solution of cyclic AMP containing 5000 cpm of cyclic [^{32}P]AMP added to monitor sample recovery. The cyclic AMP was isolated by the method of Salomon *et al.* (24). Data were calculated as percentage conversion, i.e., the percentage of total radioactive adenine taken up by the cells that was converted to cyclic AMP.

Binding experiments. Inhibition of [^{3}H]forskolin binding to bovine brain membranes was determined using a filtration assay to separate bound [^{3}H]forskolin from free [^{3}H]forskolin (10). Membranes (0.5 mg of protein/tube) were incubated for 1 hr at 20° in a total volume of 0.4 ml with 50 mM Tris-HCl buffer, 5 mM $MgCl_2$, 10 mM NaF in the presence of 10 nM [^{3}H]forskolin and indicated amounts of analogues. After incubation the membranes were rapidly filtered with a Brandel cell harvester (Brandel, Gaithersburg, MD) using GF/C filters. The filters were washed three times with 4 ml of ice-cold buffer and counted. Nonspecific binding was the amount of [^{3}H]forskolin bound in the presence of 20 μM on labeled forskolin.

Solubility of forskolin in solutions of hydroxypropyl cyclodextrins. Forskolin (1 mg) was allowed to stir for 48 hr in 0.25 ml of water or 0.25 ml of 40% (w/v) aqueous solutions of either hydroxypropyl-β-cyclodextrin or hydroxypropyl-γ-cyclodextrin. After 48 hr the solutions were centrifuged and the amount of forskolin in the aqueous solution was determined by high pressure liquid chromatography using reverse phase separation on a 4.2 mm × 25 cm Dupont ODS column with a solvent system of methanol/water (65:35). Forskolin was detected by its absorbance at 310 nm. The amount of forskolin in the samples was determined from a standard curve which was linear from 1 μg to 1 mg.

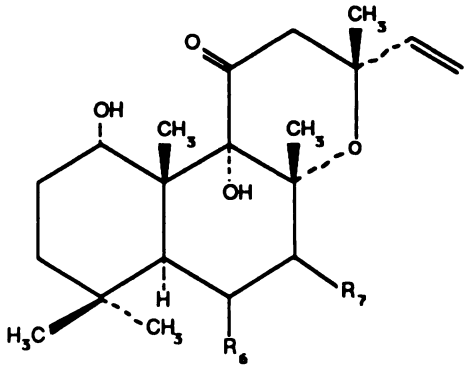
Results

Water-Soluble Analogues of Forskolin. Forskolin is a relatively lipophilic diterpene containing two α-hydroxyl groups at the 1- and 9-positions, a β-hydroxyl group at the 6-position, and a β-acetoxy group at the 7-position (Table 1). Previous studies have demonstrated that a number of acyl group substituents can be substituted at the 7β-position, and these analogues retain biological activity (25, 26). It has also been shown that 6-acyl analogues of 7-desacetyl forskolin are still relatively active, although they are less active than forskolin or 7-acyl derivatives. The water-soluble analogues described in this paper (Table 1) contain esters of heterocyclic amino acids at the 7β-hydroxyl group (**1–3**) instead of the 7β-acetoxy group which is normally present on forskolin, or at the 6β-hydroxyl position (**7–12**) of 7-desacetyl forskolin. Some analogues tested are monoacyl analogues of forskolin that contain the water-soluble ester at the 6β-hydroxyl position and the acetoxy group at the 7β-hydroxyl (**4–6**). Some of the monoacyl analogues were soluble in water at a concentration of 20 mM, whereas the other analogues required some ethanol to achieve a concentration of 20 mM. The solutions used in the experiments in this paper are given in Table 1.

Activation of rat brain adenylate cyclase. Forskolin stimulated rat brain adenylate cyclase from a basal level of

TABLE 1

Solutions of water-soluble analogues of forskolin used in the experiments



COMPOUND [1]	R_6	R_7	SOLUTION [2] (water/ethanol)
1. forskolin	OH	$OCOCH_3$	(0/1)
2.	OH	$OCO(CH_2)_3N$⟨O⟩	(1/0)
3.	OH	$OCO(CH_2)_3N$⟨NCH_3⟩	(1/0)
4.	OH	$OCO(CH_2)_4N$⟨⟩	(2/1)
5.	$OCOCH_2N$⟨⟩	$OCOCH_3$	(1/1)
6.	$OCO(CH_2)_2N$⟨⟩	$OCOCH_3$	(1/1)
7.	$OCO(CH_2)_2N$⟨O⟩	$OCOCH_3$	(1/1)
8.	$OCOCH_2N$⟨⟩	OH	(1/0)
9.	$OCOCH_2N$⟨⟩	OH	(1/1)
10.	$OCOCH_2N$⟨NCH_3⟩	OH	(1/1)
11.	$OCO(CH_2)_2N$⟨⟩	OH	(1/0)
12.	$OCO(CH_2)_3N$⟨⟩	OH	(1/1)
13.	$OCO(CH_2)_3N$⟨O⟩	OH	(1/1)

[1] All compounds were the hydrochloride salts and were dissolved in the indicated solutions to a concentration of 20 mM.
[2] The proportion of water and ethanol used to dissolve the compound is indicated.

about 120 pmol/mg/min to a stimulated level of about 800 pmol/mg/min in the presence of 100 μM forskolin. The EC_{50} for forskolin to stimulate rat brain adenylate cyclase was about 4 μM, which is consistent with the potency of forskolin to stimulate most membrane-associated adenylate cyclases. Forskolin was slightly more potent in stimulating detergent-solubilized adenylate cyclase than the membrane-associated enzyme. Forskolin stimulated the detergent-solubilized enzyme in the presence of 5 mM $MnCl_2$ from a basal level of 200 pmol/mg/min to 1300 pmol/mg/min at a concentration of 100 μM with an EC_{50} of 2 μM. The analogues were tested at concentrations ranging from 0.01 μM to 1 mM. The EC_{50} for activation of membrane and detergent-solubilized adenylate cyclase was that concentration of analogue that produced 50% of the maximal stimulation produced by 100 μM forskolin (Table 2). None of the analogues tested produced a greater stimulation of the enzyme than forskolin, although one analogue (6) was slightly more potent than forskolin.

The most potent analogues in stimulating membrane and detergent-solubilized adenylate cyclase were the 7-acyl analogues and the 6,7-diacyl analogues. The 7β-[γ-(morpholino) butyryloxy] (**2**) was slightly more potent than the 7β-[γ-(N′-methylpiperazino)butyryloxy] (**3**) and the 7β-[δ-(piperadino)valeryloxy] (**4**) analogue. However, both the 7β-[γ-(morpholino)butyryloxy] (**2**) and 7β-[δ-(piperidino)valeryloxy] (**4**) analogues were equipotent with forskolin in stimulating the detergent-solubilized adenylate cyclase. The diacyl analogue (**6**) containing the 6β-[β′-(piperidino)propionyloxy] ester at the 6-position and the acetoxy group at the 7-position was the most potent analogue with EC_{50} values of 2 μM and 1 μM for stimulating membrane-associated adenylate cyclase and detergent-solubilized adenylate cyclase, respectively. This analogue was about 2-fold more potent than forskolin. The 6β-[(piperidino)acetoxy] analogue (**5**) was equipotent with forskolin in stimulating adenylate cyclase while the 6β-[β′-(morpholino)propionyloxy] analogue (**7**) was about 2-fold less potent than forskolin.

The 6-acyl analogues of 7-desacetylforskolin were about 10-fold less potent than the corresponding 7-acyl analogues. Thus, the 6β-[γ-(morpholino)butyryloxy] analogue (**13**) had an EC_{50} of 60 μM for activating membrane adenylate cyclase, whereas the 7β-[γ-(morpholino)butyryloxy] analogue (**2**) had an EC_{50} of 4 μM for activating membrane adenylate cyclase. The EC_{50} values for stimulation of membrane adenylate cyclase by the 6-acyl analogues of 7-desacetyl forskolin ranged from 20 μM to 100 μM while the EC_{50} values for the 7-acyl analogues of 7-desacetyl forskolin ranged from 4 μM to 10 μM. The 6-acyl analogues were also less potent in stimulating detergent-solubilized adenylate cyclase than the 7-acyl analogues. It is very interesting that the addition of the acetoxy group to the 7β-hydroxyl position can enhance the potency of the 6-acyl analogues. This, the 6β-[(piperidino)acetoxy] analogue of 7-desacetyl forskolin (**8**) is 25-fold less potent in stimulating membrane adenylate cyclase than the 6β-[(piperidino)acetoxy] analogue of forskolin (**5**). The same increase in potency is also noted for the 6β-[β′-(piperidino)propionyloxy] analogue (**6**) of forskolin when compared to the 6β-[β′-(piperidino)propionyloxy] analogue (**11**) of 7-desacetyl forskolin. The 6β-[β′-(piperidino)propionyloxy] analogue (**6**) of forskolin was actually more potent than forskolin in stimulating membrane and soluble adenylate cyclase.

Many of the analogues showed a saturable stimulation of adenylate cyclase, with the activity at a concentration of 1 mM being identical to the activity at 200 μM. However, the maximal extent of stimulation by any of these compounds was never more than about 15% higher than that observed with 200 μM forskolin. One analogue (**10**) showed a biphasic effect on membrane adenylate cyclase, stimulating the enzyme at concentrations less than 10 μM and then inhibiting the enzyme at higher concentrations. It was not determined whether this inhibition of activity was due to a denaturation of the enzyme or due to the interaction of the analogue at a site different from that associated with activation. However, it should be noted that many of the analogues showed an inhibitory effect on cyclic AMP content in wild-type S49 cells at high concentrations. These inhibitory effects could be due to a disruption of membrane structure, since a marked inhibition at high concentrations was observed for many of the analogues in intact cells, whereas there was no inhibition observed for the detergent-solubilized enzyme.

Stimulation of cyclic AMP synthesis in intact wild-type S49 cells. The analogues were tested for their ability to increase cyclic AMP synthesis in wild-type S49 cells. Experiments were carried out in the presence of a phosphodiesterase inhibitor in order to maximize the amount of cyclic AMP produced. Forskolin increased the percentage conversion of adenine-labeled nucleotides to cyclic AMP from a basal level of about 0.5% to a stimulated level of about 20% with an EC_{50} of 5 μM. The analogues exhibited similar differences in potency as observed for the activation of the rat brain membrane and detergent-solubilized adenylate cyclase. The 7-acyl analogues

TABLE 2

Potency of forskolin and water-soluble analogues of forskolin

Analogues were dissolved in the solutions described in Table 1. The activation of adenylate cyclase and the increase in intracellular cyclic AMP by forskolin and the analogues were determined at concentrations ranging from 0.01 μM to 1 mM. The EC_{50} for each analogue was that concentration of analogue that produced 50% of the maximal stimulation produced by forskolin. These EC_{50} values are therefore determined relative to the maximal stimulation produced by forskolin. All determinations were carried out at least three times and the values given are the average of the triplicate determinations.

Compound	Adenylate cyclase (EC_{50})		Cyclic AMP[c] (EC_{50}) S49	Inhibition of binding[d] (IC_{50}) Human platelet membranes
	Membrane[a]	Soluble[b]		
	μM		μM	nM
1 Forskolin	4	2	5	25
2	4	2	13	58
3	13	6	12	70
4	10	2	5	31
5	4	2	18	31
6	2	1	3	5
7	10	4	23	20
8	100	30	50	300
9	75	30	63	375
10	75	20	150	93
11	23	16	75	100
12	20	30	77	214
13	60	25	75	100

[a] Rat brain adenylate cyclase was assayed as described in Experimental Procedures.

[b] Detergent-solubilized adenylate cyclase was assayed in the presence of 5 mM $MnCl_2$ in order to maximize the stimulation by forskolin.

[c] Cyclic AMP content was measured in intact S49 wild-type cells as described in Experimental Procedures.

[d] Analogues were incubated with rat brain membranes in the presence of 10 nM ^{3}H-forskolin. The IC_{50} was that concentration of analogue that inhibited 50% of the specific binding.

and the 6,7-diacyl analogues were very potent in increasing cyclic AMP with EC_{50} values that ranged from 5 μM to 25 μM whereas the 6-acyl analogues had EC_{50} values that ranged from 50 μM to 75 μM. The 6β-[β'-(piperidino)propionyloxy] (**6**) analogue of forskolin was about 2-fold more potent than forskolin in increasing cyclic AMP, consistent with its effects on rat brain membrane and detergent-solubilized adenylate cyclase. High concentrations (>200 μM) of many of the analogues led to a decrease in cyclic AMP content when compared to the levels of cyclic AMP observed at 200 μM. This inhibition was observed for forskolin, monoacyl analogues, and diacyl analogues of forskolin. Maximal levels of cyclic AMP were observed at 200 μM for most of the analogues, even though many of the less potent analogues did not increase cyclic AMP content to the maximal amount observed with forskolin or the other more potent analogues.

Inhibition of [^{3}H]forskolin binding to rat brain membranes. High affinity binding sites for forskolin have been described in rat brain membranes and human platelet membranes (10–13). These sites have a K_d of 20 nM for [^{3}H]forskolin, and the structure-activity relationship for the binding of forskolin analogues to these sites is consistent with that for forskolin activation of adenylate cyclase. Forskolin had an IC_{50} of 25 nM for the inhibition of [^{3}H]forskolin binding at a concentration of 10 nM free [^{3}H]forskolin. The 7-acyl analogues had IC_{50} values that ranged from 31 nM to 70 nM, whereas the diacyl analogues appeared to be slightly more potent with IC_{50} values that ranged from 5 nM to 30 nM. The 6β-[β'-(piperidino)propionyloxy] analogue (**6**) of forskolin was the most potent compound tested with an IC_{50} of 5 nM which was considerably lower than that of forskolin. The 6-acyl analogues of 7-desacetyl forskolin had IC_{50} values that ranged from 50 nM to 375 nM and were all less potent than either the 7-acyl analogues or the 6,7-diacyl analogues.

Potency of forskolin in aqueous solutions of hydroxypropyl-γ-cyclodextrin. The maximum solubility of forskolin in water was determined by high pressure liquid chromatography to be less than 0.15 mM. In contrast, the maximum solubility of forskolin was 2.5 mM and 9.8 mM in 40% (w/v) aqueous solutions of hydroxypropyl-β-cyclodextrin and hydroxypropyl-γ-cyclodextrin, respectively. The potency of forskolin to stimulate adenylate cyclase was determined with solutions made from dilutions of either a 20 mM ethanol solution of forskolin or a 10 mM aqueous solution of forskolin in hydroxypropyl-γ-cyclodextrin. The cyclodextrin solution did not have any effect on adenylate cyclase basal activity up to a concentration of 1%. There was no difference in potency for forskolin stimulation of rat brain membrane adenylate cyclase when dilutions were made from either the 20 mM ethanol solution or the 10 mM cyclodextrin solution (Fig. 1B). In contrast, forskolin was about 2-fold more potent in increasing cyclic AMP in wild-type S49 cells when dilutions were made from the cyclodextrin solution than when dilutions were from the ethanol solution. The maximal stimulations of membrane adenylate cyclase, detergent-solubilized adenylate cyclase, and cyclic AMP in wild-type S49 cells were identical for forskolin solutions from the ethanol stock or the cyclodextrin stock.

Discussion

Forskolin has been utilized as a pharmacologic agent in studies relating to the biochemistry and regulation of adenylate cyclase and cyclic AMP in diverse systems that include solubilized and purified enzyme preparations as well as studies in intact animals (5). Many of these investigations have been hampered by the poor water solubility of forskolin which has necessitated that forskolin be dissolved in organic solvents. Organic solvents can affect the potency of forskolin and have also been shown to decrease the efficacy of forskolin (20, 21). This has made it difficult to properly analyze dose-response relationships for forskolin where the EC_{50} for forskolin is in the μM range. Many tissues and cell preparations are also sensitive to ethanol and dimethyl sulfoxide, which have been used as solvents for forskolin. Administration of forskolin to test animals has been hampered by the high concentrations of organic solvents that are necessary to solubilize mM concentra-

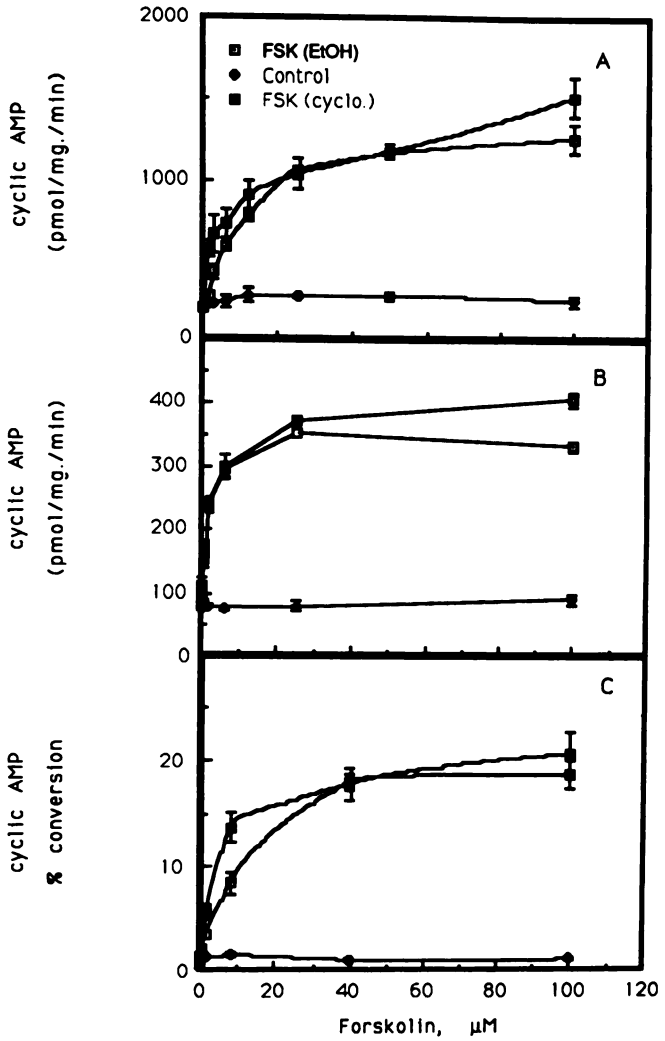


Fig. 1. Activation of adenylate cyclase by forskolin dissolved in ethanol or hydroxypropyl-γ-cyclodextrin solutions. Stock solutions of forskolin were prepared by dissolving forskolin (*FSK*) in either 100% ethanol (*EtOH*) (⊡) at a concentration of 20 mM or in a 40% (w/v) aqueous solution of hydroxylpropyl-γ-cyclodextrin (■) at a concentration of 10 mM. The indicated concentrations of forskolin were produced by diluting the stock solutions with water. Control dilutions (●) were made from a 40% (w/v) solution of hydroxypropyl-γ-cyclodextrin and were tested in order to determine the effect of the cyclodextrin solution alone on adenylate cyclase. The solutions were tested for their ability to activate rat brain detergent-solubilized adenylate cyclase (A) and rat brain membrane adenylate cyclase (B), and to increase cyclic AMP in intact S49 lymphoma cells (C).

tions of forskolin. Therefore, it would be very useful to have derivatives of forskolin that are soluble in water and which would not require organic solvents. Potential forskolin analogues should have the following characteristics in order to substitute for forskolin in biochemical and pharmacological studies. The analogues should stimulate adenylate cyclase in membrane and detergent preparations and should also increase cyclic AMP in intact cells and tissues. It has been shown that forskolin analogues that are relatively potent in activating adenylate cyclase are also relatively potent in inhibiting the binding of [^{3}H]forskolin (10). Therefore, water-soluble analogues of forskolin should have relative potencies at inhibiting [^{3}H]forskolin binding consistent with their relative potencies in stimulating adenylate cyclase.

A number of water-soluble analogues of forskolin are shown in this paper to activate adenylate cyclase with potencies and efficacies comparable to that of forskolin. The most potent analogue is the 6β-[β'-(piperidino)propionyloxy] analogue (**6**) of forskolin. This compound was found to have a greater potency than forskolin in stimulating rat brain membrane adenylate cyclase and rat brain detergent-solubilized adenylate cyclase, and in increasing cyclic AMP in intact wild-type S49 cells. The compound was also more potent than forskolin in inhibiting [^{3}H]forskolin binding to rat brain membranes. Other analogues that had potencies and efficacies comparable to that of forskolin included the 7β-[δ-(piperidino)valeryloxy] analogue (**4**) and the 6β-[(piperidino)acetoxy] analogue (**5**). It should also be noted that the 7β-[γ-(*N'*-methylpiperazino)butyryloxy] analogue (**3**) was found to be almost equipotent with forskolin in potentiating prostaglandin E_1-stimulated increases in cyclic AMP in intact platelets.[1] This analogue is now being marketed by Calbiochem. It is suggested that the water-soluble analogues of forskolin will be capable of synergistic stimulations of cyclic AMP synthesis analogous to forskolin.

The water-soluble analogues described in this paper show structure-activity relationships that are similar to those observed for more lipophilic analogues of forskolin (25, 26). It has been demonstrated that analogues of forskolin with different acyl groups at the 7β-hydroxyl position are relatively potent, whereas analogues of forskolin acylated at the 6β-hydroxyl position but lacking the 7-acetoxy group are less potent. The 7-acyl water-soluble analogues of 7-desacetyl forskolin are also more potent than the corresponding 6-acyl analogues of 7-desacetyl forskolin. Thus, the average EC_{50} for the 7-acyl analogues to activate rat brain membrane adenylate cyclase is about 10 μM, while the average EC_{50} for the 6-acyl analogues is about 60 μM. The 7-acyl analogues are also more potent than the 6-acyl analogues in stimulating rat brain detergent-solubilized adenylate cyclase, increasing cyclic AMP in S49 cells, and inhibiting [^{3}H]forskolin binding to rat brain membranes.

It is very interesting that the 6,7-diacyl analogues of forskolin are more potent than the corresponding 6-acyl-7-desacetyl analogues. These analogues may prove to be of great utility, as there should not be the possibility of acyl group migration from the 7-acyl group to the 6β-hydroxyl group as has been observed for some derivatives under basic conditions.[2] It has been reported that forskolin may be subject to enzymatic deacetylation at the 7-hydroxyl position after prolonged incubation with membranes from liver and brain (27). We have not observed such deacetylation of forskolin under any of the incubation conditions used in this paper. However, it should be interesting to determine whether the water-soluble analogues of forskolin show a different sensitivity to deacetylation than the more lipophilic analogues of forskolin.

It is difficult to draw specific conclusions concerning the size and nature of the hydrophilic portion of these molecules because of the limited number of analogues tested. The 6-acyl-7-desacetyl analogues with the lowest potency were the 6β-[(piperidino)acetoxy (**8**), the 6β-[(*N*-methylipiperazino)acetoxy] (**10**), and the 6β-[(3'-methylipiperidino)acetoxy] (**9**) analogues. These analogues had the hydrophilic portion of the ester separated from the carbonyl carbon by only one methylene group. The analogues that had the piperidine ring separated from the carbonyl carbon by two and three methylene groups (**11, 12, 13**) were slightly more potent than the acetoxy analogues. The potency of the 6β-[γ-(morpholino)butyryloxy] analogue (**13**) of forskolin was slightly less than that of the corresponding 6β-[γ-(piperidinobutyryloxy) analogue (**12**).

Hydroxypropyl cyclodextrins have been used to solubilize many lipophilic compounds in aqueous solutions (28–30). Forskolin was more soluble in 40% (w/v) aqueous solutions of hydroxypropyl-γ-cyclodextrin than in 40% (w/v) aqueous solutions of hydroxypropyl-β-cyclodextrin. The maximum solubility of forskolin in a 40% (w/v) aqueous solution of hydroxypropyl-γ-cyclodextrin was 10 mM. The cyclodextrin solution alone had no effect on cyclic AMP content in intact S49 cells or on adenylate cyclase activity in rat brain membranes or detergent-solubilized preparations from rat brain. Forskolin solutions made from stock solutions of forskolin dissolved in hydroxypropyl-γ-cyclodextrin were equipotent with forskolin solutions made from stock solutions of forskolin in ethanol in stimulating adenylate cyclase membranes, detergent preparations, and intact S49 cells. Thus, forskolin solubilized in aqueous solutions of hydroxypropyl-γ-cyclodextrin may provide an alternative to ethanol or dimethyl sulfoxide for solubilizing forskolin or when water-soluble derivatives of forskolin are not available.

It is interesting that the water-soluble derivatives of forskolin are so potent in stimulating adenylate cyclase in intact S49 cells. It was originally thought that the relative hydrophilicity of these compounds might prevent such derivatives of forskolin from crossing the membrane and acting at an intracellular site to activate adenylate cyclase. The relative potency of these analogues therefore suggested that the site of action of forskolin could be at an extracellular site. This was particularly intriguing since it is now known that some population of adenylate cyclase is glycosylated and may have an extracellular exposure (31, 32). However, recent experiments have shown that agarose derivatives of forskolin that are capable of stimulating membrane adenylate cyclase and detergent-solubilized adenylate cyclase are not capable of increasing cyclic AMP in intact cells.[3] These results are more consistent with forskolin's site of action being on the intracellular site of the membrane or perhaps within the membrane bilayer. This would suggest that the tertiary amine derivatives of forskolin used in this study, although they are more hydrophilic than forskolin, are still able to cross the

[1] A. Laurenza and K. B. Seamon, unpublished results.
[2] K. B. Seamon, unpublished results.
[3] A. Laurenza and K. B. Seamon, unpublished data.

membrane and/or interact within the bilayer at the site responsible for activating adenylate cyclase.

In conclusion, we have presented two alternatives for the use of forskolin or forskolin-like compounds in studies related to cyclic AMP in *in vitro* or *in vivo* experiments. Water-soluble analogues of forskolin can be used which retain the properties of forskolin with respect to adenylate cyclase activation. Alternatively, forskolin can be solubilized with solutions of hydroxypropyl-γ-cyclodextrin which eliminates the necessity of using organic solvents.

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