0026-895X/88/040449-05$00.00/0 $02.00/0

MOLECULAR PHARMACOLOGY, 33:449-453

Activation of Adenylate Cyclase and Inhibition of Glucose Transport in Rat Adipocytes by Forskolin Analogues: Structural Determinants for Distinct Sites of Action

HANS G. JOOST, ALAN D. HABBERFIELD, IAN A. SIMPSON, ANTONIO LAURENZA, and KENNETH B. SEAMON

Experimental Diabetes, Metabolism and Nutrition Section, MCNEB/National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health (H.G.J., A.D.H., I.A.S.) and Laboratory of Molecular Pharmacology, Division of Biochemistry and Biophysics, Food and Drug Administration, (A.L., K.B.S.), Bethesda, Maryland 20892

Received November 4, 1987; Accepted January 13, 1988

SUMMARY

Forskolin and four analogues of forskolin, 7-β-[γ-(*N'*-methylpiperazino)-butyryloxy]-7-desacetylforskolin, 7-desacetylforskolin, 7-tosyl-7-desacetylforskolin, and 1,9-dideoxyforskolin, were tested for their ability to activate adenylate cyclase, inhibit glucose transport, and inhibit cytochalasin B binding in rat adipocyte membranes. Forskolin was the most potent analogue in activating adenylate cyclase with an EC_{50} of 2 μM, whereas 7-β-[γ-(*N'*-methylpiperazino)butyryloxy]-7-desacetylforskolin and 7-desacetylforskolin were less potent, with EC_{50} values of 3 μM and 20 μM, respectively. The 7-tosyl-7-desacetylforskolin and 1,9-dideoxyforskolin did not stimulate adenylate cyclase even at the highest concentrations tested (100 μM). In contrast, forskolin and all of the analogues were able to fully inhibit glucose transport in adipocyte plasma membranes. The order of potency for the inhibition was forskolin > 7-β-[γ-(*N'*-methylpiperazino) butyryloxy]-7-desacetylforskolin > 7-desacetylforskolin > 7-tosyl-7-desacetylforskolin > 1,9-dideoxyforskolin, and the EC_{50} values were 0.24 μM, 1.8 μM, 7.1 μM, 8.8 μM, and 12.8 μM, respectively. Cytochalasin B binding to rat adipocyte membranes was inhibited by forskolin and the four analogues with the same order of potency as observed for the inhibition of glucose transport. Thus, the site of action of forskolin which is responsible for the inhibition of glucose transport and cytochasin B binding exhibits structural requirements for forskolin and its analogues that are different from those of the site responsible for the activation of adenylate cyclase.

Forskolin activates almost all mammalian adenylate cyclases and has been widely used to investigate cyclic AMP-dependent physiological responses (1). Forskolin activation of adenylate cyclase occurs primarily through a direct action of the diterpene on the catalytic subunit of adenylate cyclase (2). However, forskolin requires the presence of a guanine nucleotide-binding protein, the G_s protein, for maximal stimulation of the enzyme (3, 4). High affinity binding sites for forskolin have been described in rat brain membranes and human platelet membranes, and these sites have structural requirements for forskolin analogues that are similar to those for activation of adenylate cyclase (5, 6). It was suggested that these binding sites for forskolin are an activated complex of adenylate cyclase (6).

Physiological effects of forskolin are predominantly compatible with forskolin, acting through adenylate cyclase to increase intracellular cyclic AMP. However, there have been reports of forskolin producing effects that are not mediated by increases in cyclic AMP. Forskolin inhibits the nicotinic acetylcholine receptor-mediated influx of $^{86}Rb^{2+}$ in pheochromocytoma cells (7), blocks the outward K^+ current in pancreatic β cells from mice (8), and inhibits glucose transport in human erythrocytes, platelets, and both rat and human adipocyte plasma membrane vesicles (9–12). The binding of cytochalasin B, a potent inhibitor of glucose transport, to the glucose transporter is also inhibited by forskolin (12). These results suggest that forskolin may interact with proteins other than the adenylate cyclase enzyme system. It has also been shown that the inhibitory effect of forskolin on glucose transport in adipocytes reflects a direct interaction of forskolin with the transporter or a closely related protein (11–13).

The ability of forskolin to inhibit glucose transport raises the possibility that some physiological effects of forskolin may not be due to forskolin activation of adenylate cyclase. It was therefore of interest to determine whether there were analogues of forskolin that could distinguish between adenylate cyclase activation and inhibition of glucose transport. Lipophilic and water-soluble analogues of forskolin have been described that have different potencies for activation of adenylate cyclase (14–16). In the present study we compared the relative potencies of four analogues of forskolin to interact with the glucose transport system in rat adipocytes and to stimulate adenylate cyclase in rat adipocyte membranes.

ABBREVIATION: 7-MPB-forskolin, 7-β-[γ-(*N'*-methylpiperazino)-butyryloxy]-7-desacetylforskolin.

Experimental Procedures

Materials. Male rats (170–200 g, CD strain, Charles River Breeding Laboratories) were used throughout. Albumin (fraction V) was from Reheis Chemical Co., and collagenase (type 1) was purchased from Cooper Biochemical. All radiochemicals were from New England Nuclear. Forskolin and 7-MPB-forskolin were from Calbiochem-Behring. 7-Desacetylforskolin was prepared by alkaline hydrolysis of forskolin. 7-Tosyl-7-desacetylforskolin and 1,9-dideoxyforskolin were generously supplied by Dr. N. J. deSouza, Hoechst India.

Preparation of adipocyte plasma membranes. Rats were anesthetized with CO_2, killed by decapitation between 8 and 9 a.m., and the epididymal fat pads were removed. The adipose tissue was minced and digested with collagenase as described previously (17). Isolated adipose cells obtained from 45–90 rats were incubated for 25 min in the presence of insulin (10 nM). The cells were washed with homogenization buffer (10 mM Tris, 1 mM EDTA, 250 mM sucrose, pH 7.4) and homogenized with a Potter-Elvehjem grinder. Plasma membranes and low density microsomes were prepared by differential centrifugation as described (18).

Assay of adenylate cyclase. Adenylate cyclase activity was determined as previously described (2). Crude membranes 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-^{32}P]ATP. 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 by the method of Salomon *et al.* (19).

^{3}H-Forskolin binding. 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 (5). 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. After incubation the membranes were rapidly filtered with a Brandel cell harvester (Brandel, Gaithersburg, MD) using Whatman 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 unlabeled forskolin.

Assay of glucose transport in plasma membranes. Glucose transport in plasma membrane vesicles was determined under equilibrium exchange conditions using a filtration assay as previously described in detail (20). Samples (20–40 mg of membrane protein) were incubated in 30 ml of buffer containing equal concentrations of D-glucose and L-glucose (0.1 mM) at 22° for at least 30 min, and were pulsed for 2.5 sec with 30 μl of the same medium containing approximately 1 μCi of D-[U-^{14}C]glucose and L-[^{3}H]glucose. Uptake was stopped with a 15-fold dilution of ice-cold incubation medium containing 0.13 mM phloretin, and membranes were separated from buffer by filtration. Initial velocities were calculated after correction for L-glucose uptake as described previously (20).

Assay of cytochalasin B binding. Cytochalasin B binding was determined in the presence or absence of forskolin derivatives as indicated with the aid of a previously described binding assay (21). The assay was carried out in the presence of 2 μM cytochalasin E in order to reduce binding to sites other than the glucose transporter (21). A single cytochalasin B concentration (330 nM) and varying concentrations of forskolin derivatives were used, and the data were corrected for nonspecific binding as measured in the presence of 500 mM of D-glucose.

Results

Activation of adenylate cyclase. Forskolin and the analogues of forskolin were tested for their ability to activate adenylate cyclase in plasma membranes from rat adipocytes (Fig. 1). The rank order of potency for the forskolin analogues

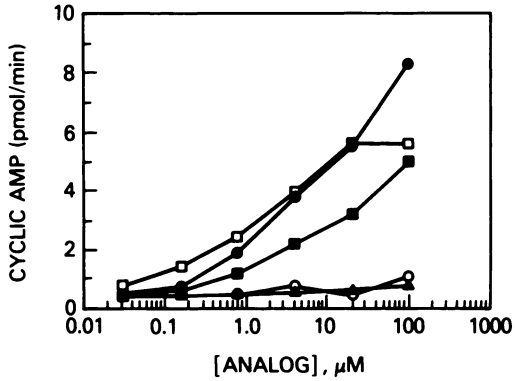

Fig. 1. Activation of rat adipocyte adenylate cyclase by forskolin and derivatives of forskolin. Rat adipocyte adenylate cyclase was assayed as described under Experimental Methods in the presence of the indicated amounts of forskolin (□), 7-MPB-forskolin (●), 7-desacetylforskolin (■), 7-tosyl-7-desacetylforskolin (▲), or 1,9-dideoxyforskolin (○).

to stimulate adenylate cyclase in rat adipocyte plasma membranes was: forskolin > 7-[γ-(N′-methylpiperazino)-butyryloxy]-7-desacetylforskolin (7-MPB-forskolin) > 7-desacetylforskolin. The 1,9-dideoxyforskolin and the 7-tosyl-7-desacetylforskolin were not able to stimulate the enzyme at any concentration and therefore do not appear to interact at the adenylate cyclase-binding site. Forskolin activated the enzyme about 10-fold over basal levels of activity with an EC_{50} of 2 μM. The water-soluble analogue of forskolin, 7-MPB-forskolin, activated the adipocyte adenylate cyclase with an EC_{50} of 3 μM, only slightly less potent than forskolin. This analogue of forskolin has a potency and an efficacy almost identical to those of forskolin for stimulation of rat brain adenylate cyclase and for increasing cyclic AMP in intact S49 cells (16). 7-Desacetylforskolin had an EC_{50} of 20 μM and was less potent than forskolin or 7-MPB-forskolin. 7-Desacetylforskolin has also been shown to be less potent than forskolin in stimulating brain adenylate cyclase (14).

Inhibition of glucose transport. Glucose transport in plasma membrane vesicles from rat adipocytes has previously been shown to be inhibited by forskolin (10–12). This inhibition was competitive with glucose and it was suggested that the inhibition of transport by forskolin was due to a direct action of forskolin at the transport protein (12). Forskolin inhibited glucose transport with an EC_{50} of 0.24 μM (Fig. 2, Table 1). All of the forskolin analogues were able to fully inhibit the transport of glucose at the highest concentration tested (Fig. 2). The rank order of potency for the analogues to inhibit transport was: forskolin > 7-MPB-forskolin > 7-tosyl-7-desacetylforskolin > 7-desacetylforskolin > 1,9-dideoxyforskolin, with EC_{50} values of 0.24 μM, 1.8 μM, 7.1 μM, 8.8 μM, and 12.8 μM, respectively (Table 1).

Inhibition of cytochalasin B binding. Forskolin has been shown to competitively displace the glucose-inhibitable component of cytochalasin B binding to adipocyte plasma membranes (12). Forskolin inhibits cytochalasin B binding with an EC_{50} of 0.27 μM (Table 1). Forskolin and all of the analogues could displace 100% of the glucose-inhibitable component of cytochalasin B binding. The rank order of potency for the analogues to inhibit cytochalasin B binding was similar to that for the inhibition of glucose transport: forskolin > 7-MPB-forskolin > 7-tosyl-7-desacetylforskolin > 7-desacetylforskolin > 1,9-dideoxyforskolin.

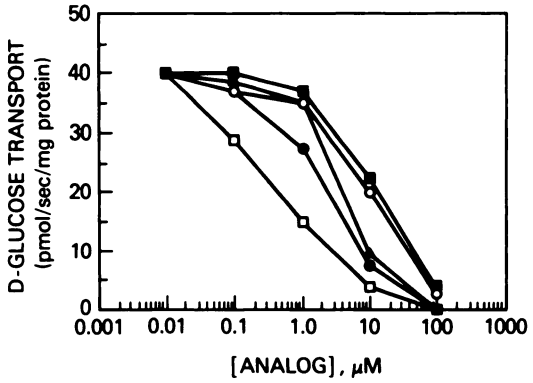

Fig. 2. Inhibition of glucose transport in rat adipocyte vesicles by forskolin and derivatives of forskolin. Glucose transport was measured in vesicles prepared from rat adipocytes as described under Experimental Methods in the presence of the indicated amounts of forskolin (□), 7-MPB-forskolin (●), 7-desacetylforskolin (▲), 7-tosyl-7-desacetylforskolin (○), or 1,9-dideoxyforskolin (■).

TABLE 1

Inhibition of glucose transport and cytochalasin B binding by forskolin analogues

	EC_{50}		
	Glucose transport[a]	Cytochalasin B binding[b]	Adenylate cyclase[c]
		μM	
Forskolin	0.24	0.27	2
7-MPB-forskolin	1.8	2.0	3
7-Desacetylforskolin	8.8	5.9	20
7-Tosyl-7-desacetylforskolin	7.1	5.2	
1,9-Dideoxyforskolin	12.8	9.7	

[a] Glucose uptake in plasma membrane vesicles was determined as described under Experimental Procedures and is the mean of three experiments.

[b] Inhibition of cytochalasin B binding was determined as described under Experimental Procedures and is the mean of two experiments.

[c] The EC_{50} is that concentration of analogue that activated adenylate cyclase in adipocyte membranes to 50% of the maximal activation observed with 100 μM forskolin.

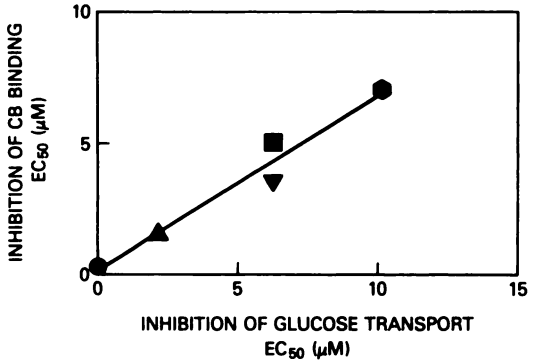

Fig. 3. Correlation between inhibition of glucose transport and inhibition of cytochalasin B binding by forskolin and derivatives of forskolin. The EC_{50} values for the inhibition of glucose transport and the inhibition of cytochalasin B binding by forskolin and derivatives were taken from Table 1. Data are plotted for forskolin (●), 7-MPB-forskolin (▲); 7-desacetylforskolin (▼), 7-tosyl-7-desacetylforskolin (■), and 1,9-dideoxyforskolin (●).

It has previously been shown that the potency of forskolin to inhibit cytochalasin B binding is similar to its potency to inhibit glucose transport (12). The ability of the four forskolin analogues to inhibit cytochalasin B binding also correlates well with their ability to inhibit glucose transport (Fig. 3).

Effect of cytochalasin B and D-glucose on forskolin binding and forskolin activation of adenylate cyclase. Cytochalasin B and D-glucose were tested for their ability to inhibit forskolin binding to rat brain membranes. Forskolin bound to rat brain membranes with a K_d of 20 nM and a B_{max} of 450 fmol/mg of protein (data not shown). Unlabeled forskolin inhibited the binding of 20 nM ^{3}H-forskolin with an EC_{50} of 30 nM (Fig. 4). There was no effect of 0.5 M D-glucose or 100 μM cytochalasin B on the EC_{50} of forskolin to displace bound ^{3}H-forskolin (Fig. 4). There was a small but significant inhibition (15%) of ^{3}H-forskolin binding by 100 μM cytochalasin B. However, lower concentrations of cytochalasin B (10 μM) did not affect the binding of ^{3}H-forskolin. The EC_{50} for forskolin activation of rat brain adenylate cyclase was also not affected by the presence of cytochalasin B (100 μM) or 0.5 M D-glucose (data not shown). These agents did not have any effect on the basal activity of adenylate cyclase.

Discussion

The physiological effects of forskolin have been almost exclusively attributed to its ability to stimulate adenylate cyclase with a resultant increase in the levels of intracellular cyclic AMP. However, studies on K^+ currents in islet cells, Rb^{2+} uptake in PC12 cells, and glucose transport in adipocytes have revealed actions of forskolin that are apparently distinct from those mediated by cyclic AMP (7–12). The aim of this study was to compare the interactions of forskolin and forskolin derivatives with the rat adipocyte adenylate cyclase and glucose transporter in order to determine whether a common binding site for forskolin exists.

Forskolin was the most potent compound in stimulating adenylate cyclase in adipose cell plasma membranes, and the hydrophilic 7-MPB-forskolin was only slightly less potent than forskolin. 7-Desacetylforskolin was approximately 10-fold less potent than forskolin, and both 1,9-dideoxyforskolin and 7-tosyl-7-desacetylforskolin were incapable of activating the enzyme, even at concentrations of 100 μM. These observations are consistent with previous results which indicated that derivatives of forskolin that perturb the α-face of the molecule had reduced potency in stimulating adenylate cyclase (14, 15). Thus, 1,9-dideoxyforskolin, 11-deoxy-11α-hydroxyforskolin, 1,9-carbonatoforskolin, and 14,15-oxidoforskolin are all extremely poor stimulators of adenylate cyclase (14, 15).

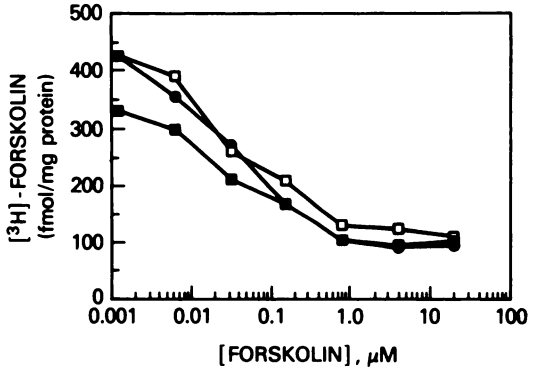

Fig. 4. Effect of cytochalasin B and D-glucose on the binding of ^{3}H-forskolin to rat brain membranes. The inhibition of ^{3}H-forskolin binding by unlabeled forskolin was determined with no additions (●), 0.5 M D-glucose (□), or 100 μM cytochalasin B (■). There was a small inhibition of ^{3}H-forskolin binding at 100 μM cytochalasin B which was not observed at 10 μM cytochalasin B.

Forskolin was shown to be a competitive inhibitor of glucose transport in both rat and human adipocytes (9–12), and, more recently, Shanahan *et al.* (13) demonstrated that ^{3}H-forskolin was specifically incorporated into the 55-kDa glucose transporter of the human erythrocyte after photolysis. Further confirmation of the interaction of forskolin with the rat adipocyte glucose transporter was provided by Joost and Steinfelder (12) who demonstrated that forskolin inhibits cytochalasin B binding to adipocyte plasma membranes. Cytochalasin B is a potent competitive inhibitor of glucose transport and is known to bind to the cytoplasmic face of the glucose transporter (22). In this study, forskolin and its derivatives were tested for their ability to block cytochalasin B binding to rat adipocyte plasma membranes prepared from insulin-stimulated cells and to inhibit specific glucose transport in the same membranes. Forskolin was the most potent inhibitor of both glucose transport and cytochalasin B binding. The other derivatives of forskolin also inhibited glucose transport and cytochalasin B binding with the same relative potencies for both processes.

Although forskolin is able to inhibit both cytochalasin B and glucose binding to the glucose transporter, there appears to be no effect of these agents on forskolin binding to adenylate cyclase. This is apparent from the lack of effect of glucose and cytochalasin B on ^{3}H-forskolin binding to rat brain membranes (Fig. 4) and their inability to inhibit forskolin activation of adenylate cyclase in rat brain membranes and human platelet membranes.[1] The inability of glucose and cytochalasin B to inhibit the high affinity binding of forskolin to rat brain membranes is consistent with these high affinity binding sites being associated with adenylate cyclase. The binding of ^{3}H-forskolin to the glucose transporter can, however, be measured using a centrifugation assay, and this binding is inhibited by glucose and cytochalasin B.[2]

The interactions of forskolin and its derivatives with the glucose transport system were clearly different from their interactions with adenylate cyclase. The most obvious differences are: 1) the ability of both 1,9-dideoxyforskolin and the hydrophobic 7-tosyl-7-desacetylforskolin to completely inhibit both glucose transport and cytochalasin B binding, and 2) the water-soluble 7-MPB-forskolin was less potent than forskolin in interacting with the glucose transport system, whereas it was almost equipotent with forskolin in stimulating the rat adipocyte adenylate cyclase.

An interesting insight into the interactions of forskolin can be obtained if the structures of glucose and forskolin are compared (see Fig. 5) and related to the binding of glucose and other sugars to the erythrocyte glucose transporter as proposed by Holman and colleagues (23, 24). Forskolin has four hydroxyl groups which are superimposable with those of α-D-galactose, a sugar which is the 4-epimer of glucose and transported with an affinity equal to that of glucose (25). The 1- and 9-hydroxyl groups of forskolin correspond to the 6- and 4-hydroxyls of α-D-galactose, whereas the 6-hydroxyl and 7-acetoxy oxygens of forskolin superimpose the 1- and 2-hydroxyls of both α-D-galactose and α-D-glucose. A model, proposed by Holman and colleagues (23, 24), for the binding of glucose to the exterior surface of the transporter is based on extensive transport

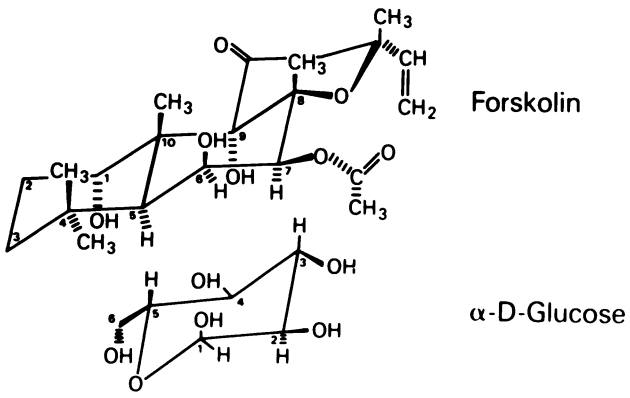

Fig. 5. Structure of forskolin and α-D-glucose. The structures of forskolin and α-D-glucose are shown with carbons *1–10* indicated for forskolin and carbons *1–6* indicated for glucose. The C-1, C-2, and C-6 hydroxyl groups of α-D-glucose are in orientations identical to those of the C-6 hydroxyl, the C-7 acetoxy oxygen, and the C-1 hydroxyl of forskolin. The epoxide oxygen of forskolin at C-8 is in an orientation similar to that of the C-3 hydroxyl group of α-D-glucose. The C-9 hydroxyl group of forskolin is in an axial orientation in contrast to the corresponding hydroxyl group of α-D-glucose at C-4, which is in an equatorial position. It should be noted that the only difference between α-D-glucose and α-D-galactose is at the C-4 hydroxyl group, which is axial in α-D-galactose.

studies with different sugar analogues. These studies have suggested that glucose interacts with the transporter through the C-1 and C-2 face of glucose with important hydrogen bonding interactions occurring between the ring oxygen and the C-1 and C-3 and, to a lesser extent, the C-6 hydroxyl groups. Derivatives of glucose at C-3, C-4, and C-6 had little effect on binding; indeed, 3-*O*-methylglucose has a higher affinity for the glucose transporter than does glucose. Similarly, substitution of a hydrophobic group at C-6 of glucose enhances the affinity at the transporter (26). The structural similarity between α-D-glucose and forskolin allows us to postulate a model wherein forskolin binds to the glucose transporter through interactions determined by the β-face of forskolin defined by the C-6 hydroxyl, C-7 acetoxy, and C-8 ring oxygens. Thus, two of the critical hydrogen bond acceptor groups at C-1 and C-3 of α-D-glucose that are important for binding to the transporter are retained in forskolin at the C-6 hydroxyl and C-8 ring oxygens. However, the ring oxygen of glucose which is important for binding to the transporter is replaced by the C-5 tertiary carbon in forskolin, and, therefore, one potential hydrogen-bonding acceptor is lacking in forskolin. The ability of 1,9-dideoxyforskolin to inhibit glucose transport is consistent with the lack of a critical role for hydrogen bonding at C-6 of the sugar. The diminished potency of 1,9-dideoxyforskolin to inhibit transport is, however, consistent with a minor role for hydrogen bonding at the C-6 hydroxyl.

1,9-Dideoxyforskolin has also been shown to inhibit the nicotinic acetylcholine receptor-mediated Rb^{2+} uptake in pheochromocytoma cells (7). Thus, for both transport systems, it would appear that the principal mode of interaction of forskolin is not mediated through interactions determined by the α-hydroxyl groups, whereas the stimulation of adenylate cyclase and the high affinity binding of forskolin to brain membranes appears to be mediated through the α-face of forskolin. Further verification of exclusivity of the effects of forskolin on glucose transport and adenylate cyclase is the absence of any effect of either glucose (500 mM), galactose (500 mM), or cytochalasin B

[1] A. Laurenza and K. B. Seamon, unpublished data.
[2] H. G. Joost, A. D. Habberfield, I. A. Simpson, A. Laurenza, and K. B. Seamon, unpublished data.

(100 μM) on the stimulation of rat brain adenylate cyclase by forskolin. α-D-Galactose and *N*-acetylgalactose also had no effect on the high affinity binding of forskolin, which is associated with adenylate cyclase, to both rat brain and human platelet membranes (data not shown).

The apparent diverse interactions of forskolin with at least three important and highly regulated membrane proteins is intriguing, and the structural similarities between hexoses and forskolin provide us with a framework to design experiments to study the topological arrangement of the different forskolin-binding sites.

Acknowledgments

K. B. S. would like to thank Dr. Peter Van Bladeren for pointing out the structural similarity between forskolin and α-D-galactose.

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Send reprint requests to: Dr. Kenneth B. Seamon, Laboratory of Molecular Pharmacology, Division of Biochemistry and Biophysics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892.